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THE UNIVERSITY OF ALBERTA
BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES
OF EXUDATE FROM FUSARIUM CULMORUM
AND ITS RELATION TO PATHOGENESIS



by

William John McPhee.

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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OF Doctor of Philosophy

DEPARTMENT ...Plant Science.....

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and
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Physiological Properties of Exudate from Fusarium culmorum
and Its Relation to Pathogenesis.....
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ABSTRACT

Exudates have been observed on a number of fungi as liquid droplets adhering to the hyphae, and detailed investigation with Fusarium culmorum shows a definite pattern to the distribution of the droplets in relation to colony morphology. This suggests the process of exudation is of physiological significance and specific properties of the droplets, absorption and re-exudation, their apparent role in spore formation and their biochemical contents support this premise. Droplets appear to be closely associated with colony aging and their properties change as this process occurs. Initially, they are transparent and water-like, but become granular and opaque, and in some instances, packed with spores as the colony develops. The sequence of droplet development and a mechanism for the release of these droplets and their function in normal physiological functioning are investigated.

The biochemical properties of the exudate collected from F. culmorum were analyzed and a more detailed look at the following components, in relation to colony age, was carried out:

- | | |
|-----------------------------|--------------------------|
| -soluble protein | -poly phenol oxidase |
| -pectolytic enzyme system | -acid phosphatase |
| -cellulolytic enzyme system | -oxalic acid |
| -protease | -soluble reducing sugars |
| - β -glucosidase | -peroxidase |

The direct involvement of F. culmorum exudate in the process of pathogenesis was demonstrated by injection of the exudate into tomato, sunflower and bean tissue. The reactions of host tissues to

the exudate were observed.

Cell wall degrading properties of the exudate were investigated using both scanning electron microscopy and thin-layer chromatography.

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CHAPTER I

INTRODUCTION

The plant pathologist must ultimately strive to completely understand plant diseases so that control can be most effective and at the same time economical. In an ideal case, he gathers enough information to give him an understanding of the host, pathogen, and environment, as well as the interactions among them. Although there are numerous examples of control without detailed knowledge of the disease or the causal agent, the most successful control is generally obtained by a thorough knowledge of the disease epidemiology. Krause et al. (1975) emphasize the importance, and often the necessity, of what might be called "pure research" on the physiology of the pathogen in order to understand its role in a particular host-pathogen interaction.

To date, much of the study in plant pathology has been aimed at the elucidation of the resistance mechanism in the host and not at the physiology involved: for example, the nutritional requirements or metabolism of the parasite. There have been numerous studies on the nutritional requirements of many fungi, yet it has been only recently that the science of fungal physiology has become sophisticated enough to deal with the specialized nutritional requirements of some of the obligate parasitic fungi. This very recent success is largely the result of research into the nutritional requirements of the rust fungi

(Bushnell, 1968; Williams et al., 1966). However, one thing that has been clear for sometime is that many fungal enzyme systems are inducible and changes in substrate, substrate levels, or age of mycelium may drastically change the kinds and levels of the exo-enzymes released by a given fungus (Brown, 1915; Christensen et al., 1951; Cooper and Wood, 1973; Singh and Wood, 1956). For this reason the physiology of invasion of host tissue by a fungal pathogen is very different from growth of that fungus on artificial medium (Bateman, 1963; 1966). It is also clear that enzymes produced in vitro, or for that matter in vivo are not necessarily functional even though substrate is available (Cole and Wood, 1961a).

Exudation is a very common phenomenon in fungi, but as with nutritional studies, is an area of fungal physiology which is largely unexplored in relation to pathogenesis. If liquids characteristically exuded by fungi have a role in the various stages of pathogenesis, they will undoubtedly have their initial opportunity to interact at the host-parasite interface. This is the most critical barrier to infection and is of utmost importance in any attempt to understand how pathogenesis is initiated and infectivity achieved. However, it is an elusive area because 1) it is so difficult to define; 2) there are structural, physical, and biochemical aspects to the process; and 3) the relationship between host and pathogen is so interrelated that separation of cause and effect becomes almost impossible. This interface is also where the host first comes in contact with fungal exudate.

This study was designed for the purpose of investigating hyphal exudation in the fungus Fusarium culmorum and its possible role in the

stages of pathogenesis. Particular consideration is given to interpretation of the results in relation to the potato storage problem "dry rot" of which Fusarium spp. are the major pathogens.

Attempts were made to 1) record the development of the fungal exudates in relation to the aging of the colony; 2) to determine the biochemical nature of the exudate associated with F. culmorum; and 3) to investigate the effects of this exudate on plant tissues.

Of particular interest during the project was the histological and biochemical similarities between cell damage in potatoes treated with exudate and the observations made on potato tissue suffering from storage dry rot.

Fungal Exudates

The presence of liquid on fungal reproductive structures has been reported numerous times. Liquid droplets have been associated with the release of basidiospores (Buller, 1958), and investigations by Knoll (Buller, 1958), of the drops of liquid excreted from the ends of pileal hairs of Coprinus ephemerus and Psathyrella disseminata, on various cystidia and the sporangiophores of Pilobolus spp. suggest that these drops contain an unidentified colloid constituent which Knoll described as mucilaginous. He also reported that crystals of oxalic acid formed at the ends of cystidia as the drops of liquid dried.

Remsburg (1940) observed liquid exudates associated with the sclerotia of Typhula spp. and noted that a crystalline residue was

formed when the liquid was dried on a glass slide. Since then, a number of reports have been published regarding the biochemical contents of the sclerotial exudates (Colotelo, Sumner, and Voegelin, 1971a; Colotelo, 1973; Cooke, 1969; Cooke, 1971; Jones, 1970). Colotelo et al. (1971b) demonstrated the presence of a "sac" enveloping the liquid droplets on the sclerotia of Sclerotinia sclerotiorum.

Exudates associated directly with vegetative mycelium have not been reported as often. Thom, in 1930, and later Raper et al. (1968), reported exudates associated with the mycelium of colonies of Penicillium spp., while in 1932 Fenner, in describing Mycotypha microspora reported the presence of "tiny water drops" adhering to the aerial mycelium. She noted that although the droplets appeared consistently, they disappeared within a day or two, and she attributed no importance to them.

Exogenous factors associated with fungal growth and the role of these factors in pathogenesis were reported in the 1800's by Ward (1888). In this paper, he illustrates a keen skill of observation and communication as shown by the following quote:

In the neighbourhood of the mycelium, e.g. at the margin of the diseased area (Fig. 3), the cell walls bounding the lacunae, and those of the epidermis and guard-cells of the stomata, are often found to be swollen and turning brown and granular (Fig. 8). This was a phenomenon which greatly puzzled me until I found that it is due to the action of a soluble ferment excreted by the fungus itself and which slowly diffuses around and kills the cells.

This quote represents an early reference in support of the production of diffusible, toxic factors which can cause host cell damage well in advance of the invading hyphae. There are ample reports to show that fungi growing in culture release degrading enzymes into their surroundings, but the mechanism by which these exogenous compounds are released is not known. However, there is a distinction to be made between the cytoplasmic extrusions described by Ward (1888), and the exudates discussed in the opening paragraphs of this review. The extrusions described in detail by Ward, have been observed for a number of fungi associated with this study and are described in more detail under "Results".

The characteristic presence of the liquid on aerial mycelium and in association with the surface hyphae indicates that a basic physiological mechanism is in operation, which results in the constant exudation of these droplets. To date, there is no explanation of the mechanism. However, recent work on the lysosomal concept in plants and fungi (Gahan, 1973; Matile, 1969; Wilson, 1973), indicate that there is an explanation to account for the intracellular source and function for these exudates.

In plant cells, the primary function of the Golgi Apparatus seems to be to synthesize polysaccharides which are then passed out to the points of cell wall synthesis; and experimentally, it has been shown that the Golgi Apparatus of dividing cells of maize epidermis produce distinct vesicles which contribute material to cell plate formation (Leech et al., 1963; Whaley et al., 1963). Similar findings were made with Phalaris root tips (Frey-Wyssling et al., 1965), and by the time the cell plate vesicles have extended to the lateral wall

of the mother cell, primary wall deposition has already begun at the centre of the plate. With few exceptions, a primary wall is laid down onto the middle lamella. The deposition of material for cell wall production requires complex enzyme activity and these enzymes are presumably associated with the very same vesicles.

Aside from this apparent role in anabolism, the lysosomes do play an important role in pathological processes in animal systems (Dingle, 1973) and this function has been related to plant systems as well (Bracker, 1971; Gahan, 1973; Wilson, 1973).

The lysosomal concept was first postulated by DeDuve (1959) and interpretation from the original system was relatively straight forward because: first, isolation of the vesicles was from liver tissue which is highly differentiated and uniform and as a result, the vesicles so obtained were uniform in age and properties. Second, the marker enzyme, acid phosphatase, was almost entirely associated with these vesicles. Because of this concentration of acid phosphatase in the vesicular fraction in these original experiments by DeDuve, the enzyme became associated with lysosomes from all sources. However, the absence of this particular enzyme has been conspicuous in many lysosome-like structures observed in both plant and fungal tissues (Dauwaulder et al., 1969; Shaw, 1966). In fact, reports on fungal lysosomes have been quite variable and in view of the range of histochemical and cellular fractionation techniques used, the variation in the physiological state of the organism, and the number of fungal species checked, discrepancies in the types of enzymes associated with these lysosomes are understandable (Matile et al., 1967; Pitt, 1968; Scott et al., 1971; Wilson et al., 1969).

In recent studies on fungi, it has been shown that lysosomes are involved in multiple roles in the synthetic processes in the cell. They are involved in septum formation in Verticillium albo-atrum, Aspergillus nidulans, Metarrhizium anisopliae (Buckley et al., 1969; Hammill, 1972; Oliver, 1972), in conidiogenesis in A. giganteus (Trinci et al., 1967; Trinci et al., 1968), in bud formation in Rhodotorula glutinis (Marchant and Smith, 1967), and in the region of the growing tip (Grove et al., 1970). In view of this evidence, the concept must be one of a lysosomal system rather than of isolated vesicles occurring at various stages and in various locations within the organism.

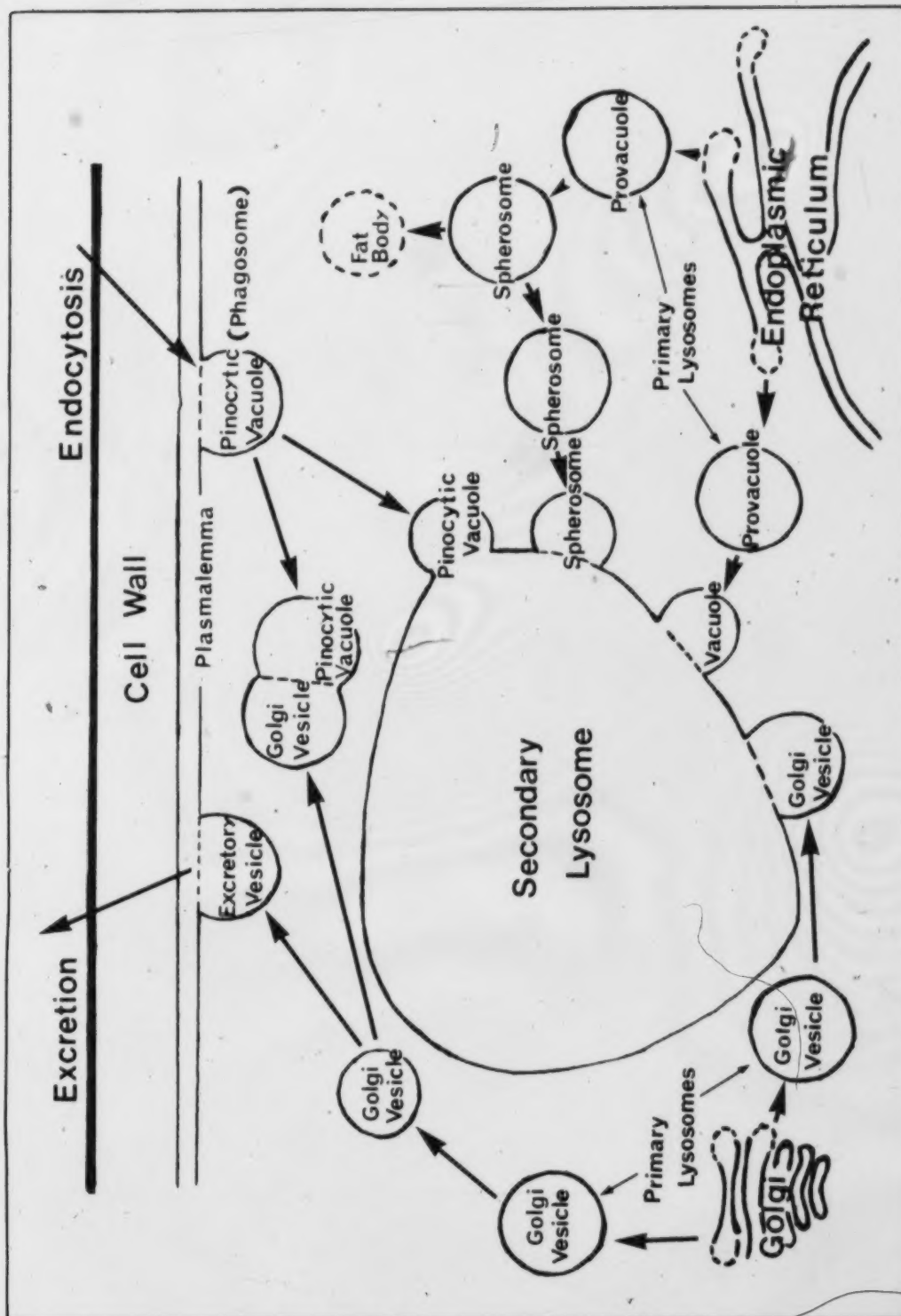
Figure 1 (from Wilson, 1973) is a schematic representation of a possible lysosomal system in higher plants and fungi, but because a Golgi Apparatus has not been found in some fungi (Bracker, 1967), the origin of the primary lysosomes in these fungi needs clarification. However, the high metabolic activity of the hyphal tip, the concentration of vesicles there, the fusion of these vesicles with the plasma membrane and the numerous reports indicating the presence of exo-acid hydrolases associated with fungal growth, all suggest that the contents of these lysosomes are extruded to the exterior of the cell as a normal consequence of fungal metabolism and physiology.

Under the conditions of shake culturing, the extruded material would be dissipated into the growth medium, and it is this type of growth procedure which is commonly used to obtain culture filtrates for the analysis of exoenzyme production by fungi. In the case of dry cultures and most still liquid cultures, the extrusion of material from aerial hyphae results in droplet formation, whereas surface and submerged hyphae result in collection of the liquid on or in the solid

FIGURE 1

A possible lysosomal system in higher plants. The Golgi Apparatus produces Golgi vesicles which may migrate to the plasmalemma and fuse with it to produce excretory vesicles. The endoplasmic reticulum also produces a number of distinct vesicles.

(From Wilson, 1973).



substrate at the periphery of, as well as surrounding the hyphal strands.

In the specific case of fungal pathogens, enough information has accumulated to allow for the formation of a concept for lysosomal behaviour in pathogenesis, and the lysosomes in both the host and parasite may play important roles in this host-parasite interaction. It is generally accepted and supported by much evidence, that the exoenzymes of the parasite may digest the host cell wall if the proper enzymes are excreted, or the reverse may be true and result in resistance to the pathogen (Abeles et al., 1970). Hydrolytic enzymes have been recorded as present in plant (Kivilian et al., 1961), and fungal cell walls (Cheung and Barber, 1971), and the initial interaction may be between their exoenzymes (Barnett, 1974; Curtis and Barnett, 1974; Strand and Mussell, 1975). It has been shown that enzymes are released from potato tissue treated with endopectate-trans-eliminase isolated from Erwinia carotovora (Stephens and Wood, 1974). Another possibility is the direct inhibition of the pathogen exoenzymes (Albersheim and Anderson, 1971), and there is evidence that these interactions may occur as the result of activation of the lysosomal system of the host and/or pathogen. For example, the migration of spherosomes into the haustorium of Piptocephalis virginiana when parasitizing Mycotypha microspora has been illustrated by transmission electron microscopy (Armentrout and Wilson, 1969). Webber and Webber (1970), report a concentration of lysosomal-like organelles in the penetration peg of a lichen haustorium of Parmelia sulcata Tayl. and they postulate that these function in providing enzymes for cell wall penetration. Pitt and Combe (1968, 1969) have evidence for lysosomal involvement in pathogenesis involving potato tubers and Phytophthora erythroseptica.

Degradation of Cell Walls

As early as the nineteenth century, DeBary (1887) was able to demonstrate and record the disorganization of host tissue using the expressed sap from plants infected with Sclerotinia sp. Ward went one step farther and extracted a similar destructive component from a Botrytis sp. In 1915, Brown introduced significant experimental improvements to the investigation of fungal "toxins" by manipulating the growth of the fungus (also a Botrytis sp.) to obtain enzyme preparations which were much more active, and therefore, more convenient to work with. Since that time, many workers have confirmed the presence of degradative enzymes in association with pathogenic fungi (Bateman and Millar, 1966; Van den Ende, 1974; Wood, 1967). Table 1 gives examples of Fusarium spp. which have been shown to produce various cell wall degrading enzymes.

Traditionally, the two enzymes associated with the breakdown of tissues have been pectolytic enzymes and cellulolytic enzymes, and the majority of research in relation to maceration has been on these two groups of enzymes. However, in reality the enzyme systems are much more complex than that. First, there are a number of pectolytic enzymes, each one specific with regards to the substrate required and to its mechanism of action. Secondly, there is also more than one cellulase involved. Thirdly, besides these two groups of enzymes, there are data supporting the involvement of specific hemicellulases (Sturdy et al., 1975), proteolytic enzymes, ligninases, cutinolytic enzymes and others (Wood, 1968). Considering the complexity of the cell wall which needs to be altered to allow effective invasion by a successful pathogen, the broad array of enzymes present or inducible

TABLE 1

PRODUCTION OF CELL-WALL DEGRADING ENZYMES BY FUSARIUM SPP.IN VITRO:

<u>Organism</u>	<u>Enzyme</u>	<u>Reference</u>
<u>Fusarium</u> sp.	Cellulase	Venkata Ram, 1957, 1959
<u>F. solani</u>	Cellulase	Etchells <u>et al.</u> , 1958
<u>F. spp.</u>	Polygalacturonase	Etchells <u>et al.</u> , 1958 Singh and Wood, 1955
<u>F. oxysporum</u>	Polygalacturonase	Waggoner and Dimond, 1955
<u>F. oxysporum</u>	Pectin methylesterase	Waggoner and Dimond, 1955
<u>F. solani</u>	Polygalacturonate trans-eliminase	Bateman, 1966

IN VIVO:

<u>Organism</u>	<u>Host</u>	<u>Enzyme</u>	<u>Reference</u>
<u>F. moniliforme</u>	Corn Stalks	Cellulase	Foley, 1959
<u>F. roseum</u> f. <u>cerealis</u>	Carnation	Cellulase	Phillips, 1962
<u>F. solani</u> f. <u>phaseolis</u>	Bean	Polygalacturonate trans-eliminase	Bateman, 1966
<u>F. solani</u> f. <u>cucurbitae</u>	Squash	Polygalacturonate trans-eliminase	Hancock, 1968
<u>F. solani</u> f. <u>phaseolis</u>	Snap Bean Hypocotyls	Polygalacturonate trans-eliminase	Papavizas and Ayers, 1966
<u>F. solani</u> f. <u>pisi</u>	Pea Seedlings	Polygalacturonate trans-eliminase	Papavizas and Ayers, 1966
<u>F. oxysporum</u> f. <u>lycopersici</u>	Tomato	Pectin methylesterase	Langcake <u>et al.</u> , 1973

in pathogenic fungi is not surprising.*

The plant cell wall is a complex structure of polymers which surrounds the cell proper, and is separated from the cytoplasm by the cell membrane. It functions to counteract the osmotic pressure resulting from the cell contents, as support for the plant, as an inter-cellular cement, and to play a complex role in plant pathogenesis. One of the major functions in this latter case is to act as a source of nutrient for the successful pathogen, and to control the production of the degradative enzymes. The biochemical nature of the cell wall is of great significance to the pathogen (Karr, 1970).

Biochemical studies support the concept that pectic substances are laid down during the early stages of wall growth (Northcote, 1963); and therefore, would be concentrated in the middle lamella area. This was illustrated for onion root tip cells (Albersheim and Killias, 1963) and for potato tuber cell walls (McClendon, 1964). These pectic substances are generally accepted as being basically polymers of galacturonic acid with varying degrees of methylation. Formerly, it was thought that pectinaceous materials were the major constituent of the cell wall matrix in all plants, but recent evidence indicates that hemicellulose may constitute up to ninety percent of the continuous amorphous phase of cell walls in some cases (Albersheim, 1965; Zaroogian and Beckman, 1968). In the cell wall proper however, (with the exception of some green algae and most fungi) the primary constituent is cellulose formed from β -1,4-linked D-glucose molecules, some 8,000 to 12,000 units long. These form linear microfibrils which are deposited in the amorphous matrix of the cell wall (exclusive of the middle lamella which is not a component of the cell wall proper) and

these microfibrils have the capacity to become displaced from one another, an important fact when considering the mechanism of primary cell wall growth or breakdown. The primary wall itself, can be considered more like the amorphous middle lamella than the closely packed secondary wall which has as much as 50 percent cellulose fiber, i.e. in tracheids. The primary wall is also distinguished from the secondary wall by its ability to extend as the protoplast expands, by a dispersed texture of the microfibrils, and by the ability of the cell wall to show reversible changes in thickness. (The transition of material from the primary wall to secondary wall is so gradual that distinction between the two is really arbitrary.)

Fig. 2 shows the components of the primary and secondary walls and illustrates the presence of other minor components besides cellulose and pectin. The major one is hemicellulose, which consists of the sodium or potassium hydroxide soluble polymers made up predominantly of D-galactose, D-mannose, L-arabinose, D-xylose, L-rhamnose and uronic acids (see Table 2 and Fig. 3). Typically, α -cellulose is an unbranched glucose polymer whereas the hemicelluloses are branched polymers. The hemicelluloses probably occur in an amorphous condition between the microfibrils and their concentration increases toward the middle lamella. The protein content is relatively low, particularly in younger cell walls, and its role in cell wall structure is still unresolved.

Table 3 shows some typical pectin compositions in various tissues. It is apparent that pectic substances are generally rich in galacturonic acid, but also, that they contain significant amounts of neutral sugars. However, pure galacturonans do occur, but infrequently.

TABLE 2

CARBOHYDRATE POLYMERS OF PLANT CELL WALLS

<u>Polymer</u>	<u>Known Component(s)</u>
1. CELLULOSE	D-glucose
2. HEMICELLULOSE	Xylans Glucomannans
3. PECTIC Substances	Galacturonans Arabinans Galactans and/or arabinogalactans I* or II
4. OTHER POLYSACCHARIDES	Arabinogalactans II Fuco- (or galacto-) xyloglucans
5. GLYCOPROTEINS	

- * Arabinogalactans of type I are characterized by essentially linear chains of (1-4) linked β -D-galactopyranose residues whereas those of type II contain highly branched interior chains with (1-3) and (1-6) intergalactose linkages.

(From Aspinall, 1973)

FIGURE 2

A schematic representation of the cell wall showing the major constituents and their relative distribution within the primary and secondary walls and the middle lamella. The concentrations of components increase in the direction of the arrows.

(From Wood, 1967)

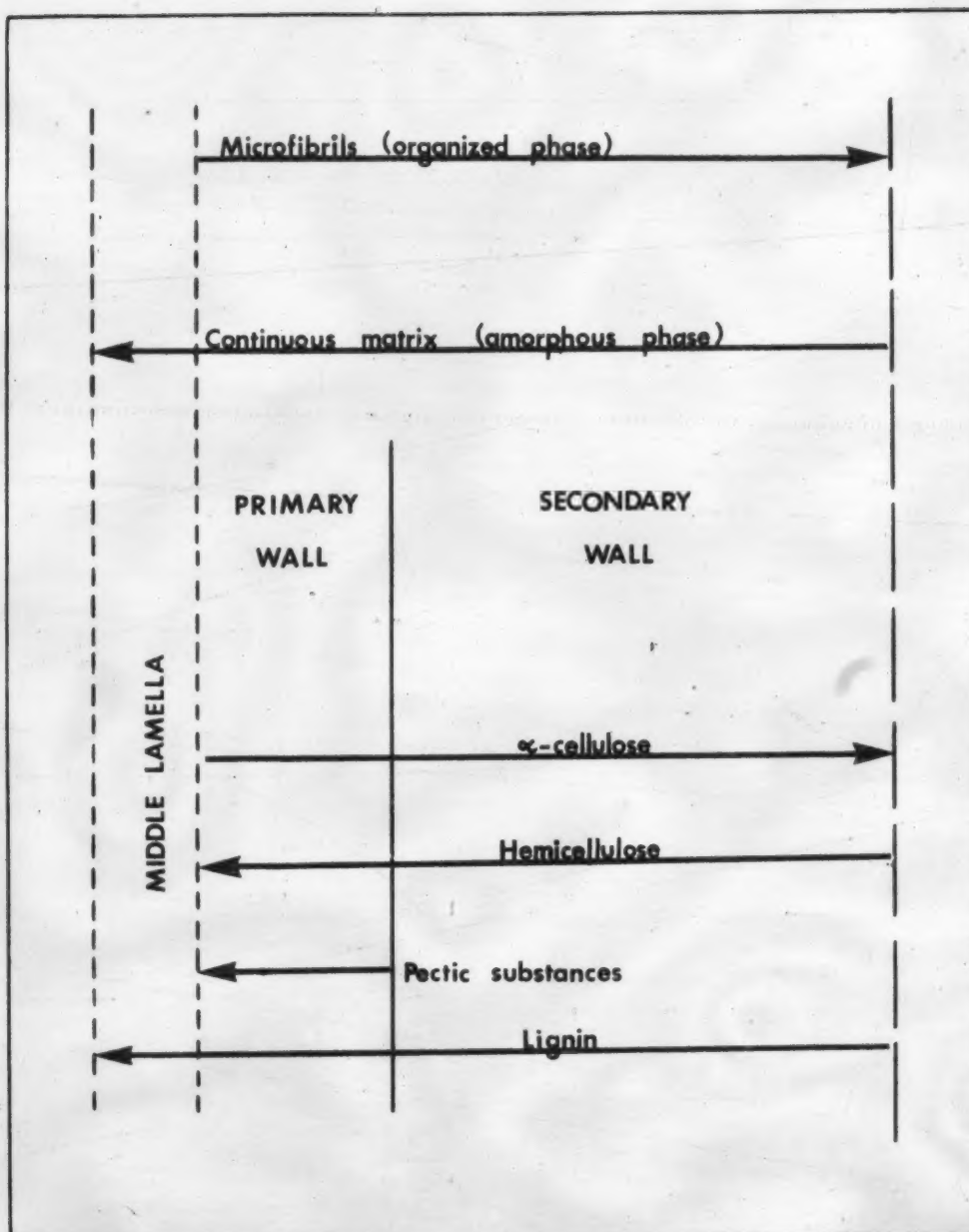


FIGURE 3

Structural formulas for some of the common cell wall mono- and polysaccharides.

- 1 = galacturonic acid
- 2 = pectic acid (polygalacturonic acid)
- 3 = methylated galacturonic acid
- 4 = pectin (methylated polygalacturonic acid)
- 5 = xylose
- 6 = xylan (a hemicellulose)
- 7 = mannose
- 8 = galactose
- 9 = rhamnose
- 10 = arabinose
- 11 = glucose
- 12 = cellulose

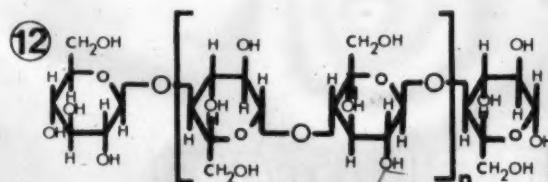
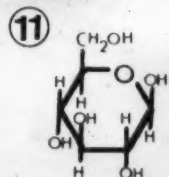
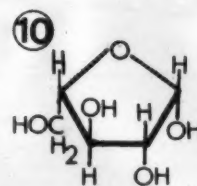
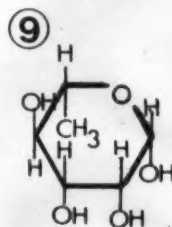
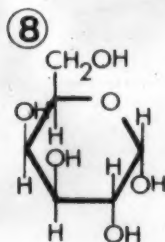
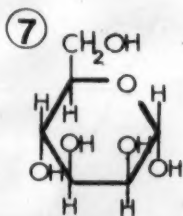
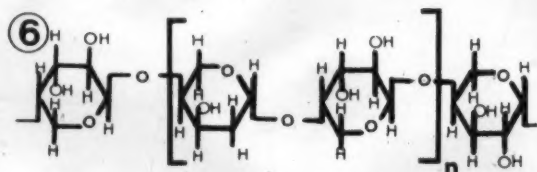
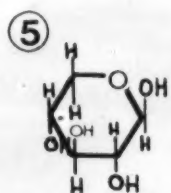
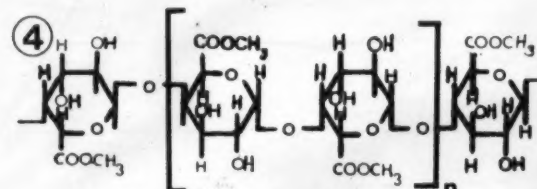
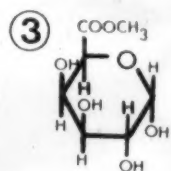
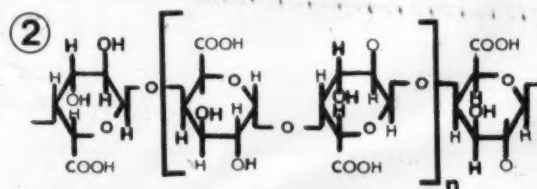
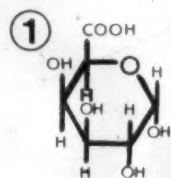


TABLE 3

THE COMPOSITION OF SOME PECTINS OF VARIOUS SOURCES

Pectin Source	Sugar Constituents					
	GalA	Gal.	Ara.	Rha.	Xyl.	Apiose
Apple	87%	1%	9%	1%	1%	
Lemon Peel	84%	+	+	+	+	
Alfalfa	81%	+	+	+	+	
Anabilis Fir Bark	77%	6%	17%	+	-	7-38%
Duckweek (<u>Lemna minor</u>)	53-80%	-	-	-	-	
Mustard Cotyledons	28%	8%	44%	3%	17%	
Soybean Cotyledons	24%	+	+	+	-	
Potato Tuber Cell Wall	25%	47%	+	+	2%	+

(From Aspinal, 1973)

One such pure galacturonan has been demonstrated in sunflower heads (Zitko and Bishop, 1966).

The neutral sugar L-rhamnose, as far as is known, occurs in pectin only, in the interior chains and appears to cause kinking of the otherwise regular chain (Rees and Wight, 1971).

Pectinic side chains of xylopyranose residues, alone or with appended D-galactopyranose or L-fucopyranose units, are found in tissues with potential for rapid enlargement and/or rapid differentiation such as pollen (Bouveng, 1965), soybean cotyledon (Aspinall, *et al.*, 1967), and white mustard seed (Rees and Wight, 1969), but are also minor constituents in more typical pectins (Aspinall *et al.*, 1968; Aspinall, Gestetner, Malloy and Uddin, 1968). Side chains containing D-galactopyranose and L-arabinofuranose residues are more characteristically typical of pectins.

The extent of the homopolysaccharide in the pectic substances of the cell wall is not well established. Much of the information may be misleading or not strictly comparable because of the variety and types of isolation procedures used. Strongly acidic conditions will result in hydrolysis of the acid-labile glycosidic linkages within the cell wall structure, and pectins with a high degree of methylation (or generally, esterified D-galacturonic acid residues) are susceptible to base-catalysed degradation (Aspinall, 1973). This latter reaction leads to the cleavage of glycosidic bonds and the formation of unsaturated hexuronic acid units. This elimination in the presence of the appropriate ionic species may occur slowly at an acid pH (Aspinall and Cottrell, 1970).

Barrett and Northcote (1965), have shown that apple pectin heated in buffer at pH 6-8 is degraded into two distinct polysaccharide fractions (Fig. 4) which show evidence for the distribution of a neutral sugar-galacturonic acid residue widely spaced along the macromolecular chain.

Cell wall pectins are generally assumed to consist of an un-oriented matrix between the cellulose fibrils of the wall; yet, as early as 1951, there were indications that pectin may appear in cell walls as fibrils (Roelofsen and Kreger, 1951; 1954). More recent recognition of the fibrils of poly-galacturonic acid on the surface of cultured cells of several species has been reported (Leppard *et al.*, 1971). White (1967), reported fibrillar aggregates during examination of cell cultures of Picea glauca, Sutton-Jones and Street (1968), observed fibrils on the surface of cultured cells of Acer, and Halperin and Jensen (1967), saw them on the outside of cells of Daucus and interpreted them to be cellulose. There seems to be no definite width to the fibrils and they sometimes appear to be split longitudinally or branched, suggesting a bundle-like structure.

These fibrils in cell culture associate between cells or on the surface of cells to form sheets which in ordinary tissue, would be proximal to the part of the cell equivalent to middle lamella (Leppard *et al.*, 1971; Leppard and Colvin, 1971; 1972).

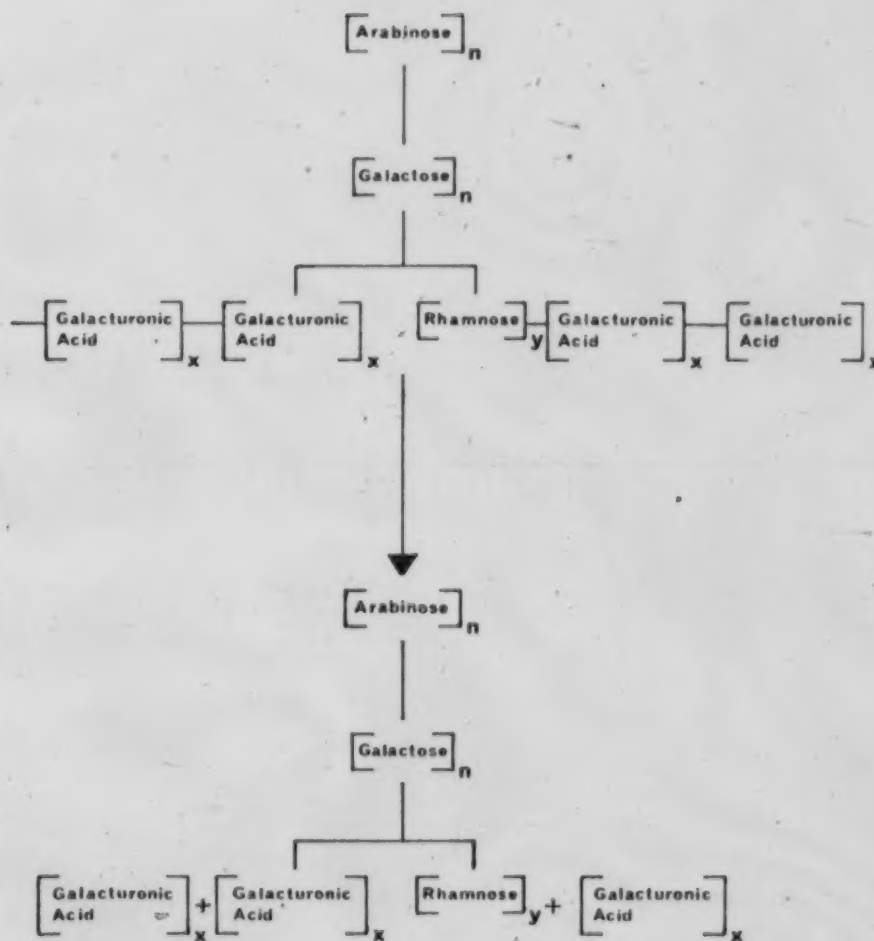
Chemical analysis of the fibrils of Ipomoea (Colvin and Leppard, 1973), show them to be 68 percent hexuronic acid. Galacturonic acid appeared as the monomer of a long-chain polymer, and no other uronic acid nor neutral reducing sugars were detected. One surprising fact was that these fibrils were resistant to pectinase

FIGURE 4

The formation of two chemically distinct polysaccharides on random degradation of pectin containing an uneven distribution of neutral sugars. The two products are (i) polygalacturonic acid and (ii) a complex consisting of galacturonasylrhamnose covalently linked to an arabinan-galactan side chain.

(From Aspinal, 1973)

(PECTIN)



(Leppard and Colvin, 1971), but these authors suggest that the presence of protein and other molecular groups may interfere with enzyme hydrolysis.

This chemical evaluation and the interpretations on plant pectins are relatively new work and their major importance is possibly as a warning that cellulose can no longer be regarded as the sole fibrillar component in plant cell walls. More care will be needed in the identification of these fibrils in the future.

Pectic substances constitute the major component of the cell wall in potato (Hoff and Castro, 1969). Table 4 illustrates the composition of potato cell wall, the specific sugar composition of the pectic materials and the sugar composition of the hemicelluloses. This work agrees with the findings of Le Tourneau (1956), but disagrees sharply with a more recent paper by Vechner and Prokazov (see Hoff and Castro, 1969). These authors found tuber cell walls to be composed of from 65 to 80 percent glucose, whereas the findings listed here (Table 4) show galactose as the major single monosaccharide.

Bettelheim and Sterling (1955), showed that the extraction technique used influences the nature of the pectinic products released from potato cell wall, and they emphasize the importance of calcium in binding the cell wall pectins and somewhat, controlling their release under various extraction conditions. Hoff and Castro (1969), have also reported a non-uniformity in the pectic fraction of potato cell walls. They point out that the large amounts of neutral sugars present will have a significant effect on the physical properties of the constituent pectic polymers and thus, on the tuber cell walls

TABLE 4
COMPOSITION OF POTATO TUBER CELL WALL

Tuber Cell Wall Content and Composition		Composition of Pectic Material		Composition of Hemicellulose	
%	(%)		%		%
Cell Wall Content		Polygalacturonic Acid	51.4 ± 10.1	Polygalacturonic Acid	6.6 ± 1.1
-Wet basis	1.2 ± 0.1	Methoxyl Content of PGA	6.2 ± 0.9	Sugar Composition:	
-Dry basis	5.6 ± 0.6	Sugar Composition:		-Rhamnose	0.5 ± 0.1
Cell Wall Composition (Dry Basis)		-Rhamnose	6.0 ± 2.1	-Fucose	0.0
-Pectic Substances	55.0 ± 7.5	-Fucose	0.6 ± 0.3	-Arabinose	2.1 ± 0.5
-Hemicellulose	6.8 ± 1.4	-Arabinose	5.6 ± 3.5	-Xylose	23.1 ± 1.1
-Cellulose + Lignin	27.5 ± 2.5	-Xylose	1.8 ± 1.1	-Mannose	5.8 ± 1.4
-Protein (N x 6.25)	9.8 ± 1.7	-Mannose	0.0	-Galactose	12.0 ± 1.2
		-Galactose	86.6 ± 5.3	-Glucose	56.7 ± 1.8

The 7% glucose found in this fraction was assumed to stem from starch contamination.

(From Hoff and Castro, 1969)

themselves. For example, the pectic material they isolated was soluble whereas normally, pectinic acids completely precipitate out of 20 percent alcohol solution. These same authors used oxalic acid-ammonium oxalate to solublize their pectic substances, but did not indicate that calcium was involved. They also speculated that there may be an araban-galactan fraction associated with the polyuronides. An arabanase-galactanase complex has been shown to be operative in carrot tissue (Hatanaka and Ozawa, 1965; see Hoff and Castro, 1969).

Although the above does not constitute an exhaustive review of the structure of the cell wall, it does point out that differences in the degree of esterification of the D-galacturonic acid residues, and the variation of sugar composition are both important in the overall assessment of cell wall pectins.

The cellulose found in primary and secondary walls appears to result from different biosynthetic pathways. This is concluded from the facts that the molecular size of the secondary wall cellulose is a constant $\bar{P}_w$ 13000 to 16000 and is independent of the extent of conversion, whereas cellulose of the primary wall is of lower polymer size and of non-uniform distribution (Marchessault and Sarko, 1967; Rees and Skerrett, 1968). Marx-Figini (1969) suggests that the molecular size of the secondary wall cellulose is genetically determined, but that morphology and crystal structure are dependent on the molecular properties of cellulose itself.

The hemicelluloses of lignified tissues fall into two groups (Fig. 3) (Timell, 1964, 1965). Only small differences in the structure of the backbone chains within each group have been detected; the major variation in structure is in the number, nature and mode of

attachment of the sugar residues and of other constituents such as O-acetyl groups, which are attached as side chains. These differences reflect differences in biological function. Generalized structures of these two groups are shown in Figure 4. Polysaccharides of the xylan group and sometimes of the glucomannan group are components of the secondary wall.

Although traditionally, attention has been directed at the polysaccharide composition of cell walls in the evaluation of tissue breakdown, the possible role of glycoproteins cannot be overlooked. Evidence does exist for sugar-amino acid linkages within the cell wall (Lamport, 1973). Some of the common sugar constituents of these glycoproteins which have been identified are arabinose (Heath and Northcote, 1971), galactose (Monro *et al.*, 1972), rhamnose (Pusztai and Watt, 1969), and galacturonic acid (Pusztai *et al.*, 1971). The biological function of these glycoproteins in the cell wall is still largely unresolved.

Since fungal enzymes capable of the breakdown of most of these components of the cell wall have been detected, it is clear that the process of invasion of host tissue by fungal pathogens is a complex one.

Tissue Degradation

Cell wall dissolution of one kind or another must accompany the invasion of host tissue, and it is clear that the process of invasion will vary with the combination of host and pathogen involved. Yet despite the potential for variation and complexity, some generalities do occur. Obligate parasites such as rusts and downy mildews grow in

the intercellular spaces or between cells when these are in contact, and penetrate intracellularly by means of short haustoria which extend into the lumen of the cell. Facultative saprophytes such as the smut fungi are also basically intercellular within host tissue, and the fact that these fungi do grow between cells without intercellular spaces indicates that there are some modifications of the cell wall. However, by light microscopy, there is little evidence of any change having occurred in the wall structure (Wood, 1967), and there appears to be no reduction in the coherence between adjoining cells because infected tissues are of the same consistency as normal tissues.

A second general type of tissue alteration occurs with the disease of parenchyma tissue resulting from attack by facultative parasites (although under certain conditions some are intercellular, e.g. *Rhizoctonia solani*). This type of breakdown is described as soft rot because the tissue becomes soft and has a waterlogged texture. This implies that two things occur: 1) an alteration in cell permeability takes place and, 2) since the cells become separated from one another, degradation of material between adjacent protoplasts occurs. There are in fact, some suggestions that macerating activity and toxicity can be completely separated. Tribe (1955), showed that maceration of tissue could occur without killing of the cells if substances in the external solution are kept slightly hypertonic, and it may be that the killing of protoplasts during maceration is the result of osmotic effects. This work was confirmed on the same experimental system by Fushtey (1957), but attempts to separate the factors responsible for maceration and cellular death failed.

Kamal and Wood (1956), reported some separation of the macerating and killing activities for enzyme extracts from Verticillium dahliae, and Basham and Bateman (1975), working with Erwinia chrysanthemi which produces a homogeneous endopectate lyase, showed both electrolyte loss from cells and cell wall breakdown in tobacco pith cells. They were able to protect the protoplasts from injury by plasmolysing the tissue prior to treatment with enzyme, yet cell wall breakdown was not retarded. Spalding (1969), indicated that tissue maceration and cellular death of sweet potato attacked by Rhizopus stolonifer are linked.

Changes in the permeability characteristics of plant cell membranes are common in diseased tissue (Wheeler and Hanchey, 1968), and it is commonly evaluated as the extent of ion leakage from diseased tissue.

The third general class of rot, again characteristic of facultative parasites, is dry rot and the significant characteristics of this disease syndrome are slow development, dry texture and (except under conditions of high humidity) a lower water content than in normal tissue. With this rot, degradation of cell walls occurs and the difference from a soft rot is that soft rot retains water while the dry rot does not.

Wood (1967) points out, "Almost all of what is known about breakdown of cell walls by plant parasites comes from the study of soft rots.", and perhaps this is not unexpected considering the importance of maceration to pathogenesis and the ease with which the symptoms of soft rot can be studied in vitro.

Dry Rot in Potato

As early as 1908, Pethybridge and Lafferty (see Boyd, 1952), reported on the susceptibility of potato tubers to dry rot and since that time, the majority of the work on dry rot has been in relation to the incidence of the disease in the field and to control of the disease in storage, while almost no work has been done on the physiology of the disease.

A number of organisms have been associated with dry rot, although Fusarium caeruleum remains the major organism causing the disease. Other species of Fusarium which have been implicated as the causal organism of dry rot are F. avenaceum (Moore, 1945), F. arthrosporoides, F. tricinctum (McKee, 1952), F. solani (Goss, 1940), F. sulphureum Schlecht (Boyd and Tickle, 1972), F. culmorum and F. sambucinum (Booth, 1971), and F. roseum 'Avenaceum' (Jones et al., 1968).

From the research done thus far, the following general facts about the disease have been established: 1) water conditions play an important role (Fernando and Stevens, 1952; Greg, 1952; Lapwood, 1957; Murant and Woods, 1957), i.e. dry rot outbreaks frequently follow severe dry seasons. However, Boyd (1967), notes that the variety Home Guard loses water faster than variety Catriona, yet the latter variety is much more susceptible. Also, Moore (1945), states that high humidity favoured rotting but that the extent of the rotting varied with the species of pathogen; 2) susceptibility is largely influenced by the variety of potato attacked; 3) there is higher susceptibility at lower temperatures (Griffin, 1964), and in cold storage. This may be due to a lag in wound periderm formation (Steward, 1943). However, Boyd (1952), found higher susceptibility at 59° F. than at 39° F. when

stored before treatment. The influence of temperature during storage may be on both the host and the pathogen.

Details of the physiology of this disease are very sparse and it is difficult to put forth any general hypothesis related to the disease syndrome on the evidence that is available. Singh and Mathus (1937), and Appleman and Miller (1926), report rapidly growing tubers in post-flowering period show a high sucrose/hexose ratio which falls off sharply when leaves begin to die. This may be correlated with Boyd's findings (1952) that there is a very high peak of susceptibility in immature tubers in the post-flowering period and a sudden decrease in susceptibility when the haulms are dead. This high susceptibility when tubers are immature and low susceptibility when lifted was reported again in 1967 by Boyd, Pethybridge and Laffery (see Boyd, 1952), discount the action of sugar concentration in influencing infection since susceptibility was not increased by low temperature storage before inoculation, which should have increased the reducing sugar content (Sereno *et al.*, 1957). Boyd (1952), suggested that pre-maturity susceptibility probably depends not on sugar but on the presence of a transitory compound which is concentrated during active growth of the tubers and which is then converted as the tubers mature. He makes no prediction as to what the transitory compound might be but one possibility is a phenolic of some kind.

Some workers have suggested that susceptibility and resistance to dry rot are related to the production of phenols by the host tissue, either by constitutive means or as a result of fungal inducement (Griffin, 1964). The classic example involves infection of onions by Colletotri-

chum circinans. This fungus is soil borne, penetrates the outer tissue of the onion by formation of an appressorium and parasitizes the inner, thick, fleshy leaves. Varieties of onions with pigmented outer scale leaves are resistant while varieties with colourless scale leaves are susceptible. The toxic pigments that account for the fungal toxicity are flavones which also occur as glycosides in plant tissue (Walker and Stahmann, 1955). Similar work has been done relating to Helminthosporium carbonum (Kuc et al., 1955; Kuc et al., 1956; Kuc, 1957) and Streptomyces scabies (Johnson and Schaal, 1952; Schaal et al., 1953; Schaal and Johnson, 1955), and in cotton in relation to Rhizoctonia solani (Hunter, 1974).

In potato tissue chlorogenic acid tends to accumulate around injured tissue in infection with S. scabies (Johnson and Schaal, 1952; Politis, 1948). Bate-Smith (see Griffin, 1964), reported that fresh potatoes are less susceptible to rot caused by F. caeruleum (Lib.) Sacc. than stored potatoes and that fresh tubers have a higher content of chlorogenic acid. But, Kuc (1957) found that although potato tissue infected with F. solani f. radicicola (F. caeruleum ((Lib.)) Sacc.) produced chlorogenic acid, the fungus was not inhibited by extracts of inoculated tuber tissue nor by high concentrations of chlorogenic acid (1×10^{-2} M.).

However, most of the phenols are very reactive; therefore, resistance is more likely due to a phenol derivative, if indeed the phenolics are involved at all.

Ultrastructural studies of cells undergoing a hypersensitive response show that the walls of the plant cells surrounding the hypersensitive cells are altered (Friend et al., 1973; Klarman and Corbett,

1974; Mercer et al., 1974; Tomiyama, 1967). Albershiem et al. (1969) offers a hypothesis to explain resistance in the true bean Phaseolis vulgaris to Colletotrichum lindemuthianum which implicates the level of glucose as a controlling factor for the production of cell wall degrading enzymes. Other workers have shown the involvement of an elicitor of fungal origin which stimulates the production of a phytoalexin and imparts resistance to the fungal attack (Ayers, A., J. Ebel and P. Albersheim. See P. Albersheim and A.J. Anderson-Prouty, 1975). Ayers et al. have shown that the elicitor is a polysaccharide and has extracted active elicitor from the purified mycelial walls of Phytophthora megasperma var. sojae. The polysaccharide portion of the elicitor preparation are 3-linked, 6-linked, 3,6-linked and terminal glucose. The structure of these elicitor polysaccharides resemble closely the non-cellulosic glucan of this pathogen's mycelial walls (Zevenhuizen, 1969).

This research has put a new emphasis on the involvement of polysaccharides in pathogenesis. First of all this will naturally implicate fungal cell wall degrading enzymes which are capable of releasing polysaccharides from host cell walls (Hancock, 1967). The effect of length of growth period and nutrition available upon tuber susceptibility to dry rot caused by Fusarium caeruleum has been studied by Boyd (1952), and he found that shortening the growth period reduces dry rot. His conclusion was that susceptibility in immature tubers was closely related to sucrose content which suggests that some kind of possible interaction between the pathogen and the host sugars. Only little work has been carried out to-date, in relation to specific sugar releasing enzymes and their role in pathogenesis, and particularly in relation to dry rot.

Sturdy and Cole (1975), have shown that the cell wall degrading α -1,3-arabinofuranosidase produced by Fusarium caeruleum is "most" active in lesions produced in susceptible potato tubers; however, they conclude that this particular enzyme is not a major factor in the process of tissue disintegration, but is a minor contributor to the process.

Elucidation of the details of rotting processes will undoubtedly be aided by the current and future work in plant cell wall structure and in the systematic, enzymatic degradation of these walls.

CHAPTER II

MATERIALS AND METHODS

Maintenance of Culture

The culture of Fusarium culmorum used is an Alberta isolate originally supplied by Dr. A. W. Henry and a stock of this culture was kept on a potato-dextrose-agar slant culture (PDA or PDA-D). The potato-dextrose-agar designated as PDA-D was prepared from commercial (Difco) potato-dextrose-agar, and that designated as PDA was prepared in the laboratory as follows:

- (i) 200g of diced potatoes in 800ml of distilled water were autoclaved at 121° at 16 psi for fifteen to twenty minutes;
- (ii) the autoclaved material was filtered to remove the potato, and the liquid extract so obtained was mixed with 20g of dextrose and 13g of bacto agar. The volume was adjusted to one litre with distilled water;
- (iii) this solution was autoclaved for twenty minutes.

These stock cultures were transferred to fresh slants every four months.

Inoculation of Plates

First, to observe droplet development in relation to colony aging, glass petrie dishes containing standard PDA-D were centrally inoculated with 4mm square plugs of inoculum and incubated at 20°. The inoculum was taken from the edge of a seven-to-fourteen-day-old colony to ensure that all stages of maturation occurred within the time and space limitations of the experiment. Second, for collection of exudate, glass petrie dishes were used. A sterilized 5 x 6 cm sheet of dialysis membrane (cellulose tubing, 0.0025 cm wall thickness, M.W. cutoff of 12,000 supplied by Arthur H. Thomas Company) was placed on the surface of the agar, inoculated centrally with a 4 mm. square plug of inoculum and stored at 24°. For these experiments only the peripheral mycelium of one-to-two-day-old colonies was used to ensure that no pigment was transferred. This was important because there is an aging factor associated with the pigment, and without this factor, mainly exudate-producing, aerial mycelia were generated which means a greater number of droplets were formed.

The purpose of the dialysing membrane was two-fold: 1) it aided in the separation of the aerial mycelium from the surface of the plates, and 2) it enhanced the amount of aerial mycelium produced, which in turn, increased the number of droplets of exudate.

Observations of Droplet Development

Colonies were observed by using incident and reflected light microscopy. There were no difficulties in observing the initial steps of mycelial development and droplet formation because the amount of mycelium was relatively sparse. Due to rapid growth, older areas of

the colony were soon covered with secondary mycelial growth and conidial masses. To allow observation of any one area of the colony, it was necessary to use 1/10 or 1/100 dilution of the media. Using this type of technique resulted in relatively reduced secondary growth and allowed for observation over long periods of time, even though linear growth had extended to the limits of the petri plate. This technique was used to obtain sequences of growth and droplet formation which were recorded photographically over several days.

Collecting the Exudate

The simplest technique for collecting the exudate was to draw the material up under slight vacuum into a fine capillary tube with an ID of 50 μ . This method was too time consuming for collection of the large quantities necessary for rapid detailed biochemical testing so the following technique was used:

The aerial mycelia were removed from the colony by means of a stainless steel spatula and deposited in a plastic centrifuge cup equipped with a false bottom. The mycelia were then centrifuged at 3400 rpm (approximately 1400 X g) on a Sorval RC2-B refrigerated centrifuge for forty minutes. The exudate which collected at the bottom of the tube was passed through a 0.45 μ m Millipore filter and the filtrate used directly for biochemical analysis. For experiments involving plant tissue, filtration was carried out under sterile conditions to avoid contamination of the tissue during injection.

Biochemical Tests

1. Total Protein:

The total protein content of the exudate was measured directly by the procedure of Lowry et al., (1951). This method is based on the formation of a copper-protein complex formed in alkaline solution which reduces the Folin reagent and produces a blue colour. The colour was read on a spectrophotometer at 630 nm and the protein quantitatively determined against a bovine serum albumin protein standard.

2. Qualitative Determination of Protein:

Electrophoretic separation and detection of protein was carried out by the method of Davis (1964). The total protein pattern was determined by staining the gel with 1 percent Amido-Schwartz dye in 7% acetic acid. The excess dye was removed by rinsing the gel in 2% acetic acid then washing overnight in 7% acetic acid.

3. Enzyme Assays:

i) Peroxidase and Polyphenoloxidase

Electrophoretic gels were also tested with the following specific dyes to determine the presence of individual enzymes:

A) Peroxidases with (a) 1 percent pyrogallol in 4 percent hydrogen peroxide.

(b) 0.5 percent benzidine dihydrochloride dissolved in a solution of 100ml of 7 percent acetic acid containing 16g of sodium acetate (tri-hydrate) and saturated with versene (EDTA).

B) Polyphenoloxidase with 0.1 percent dihydroxyphenylalanine in 0.05M phosphate buffer at pH 6.0 (Macko et al., 1967).

Protein determinations were carried out on all of the filtered exudates used in the following enzyme assays, in order that specific activities could be determined (expressed in units of enzyme activity per mg protein, when desired.)

ii) Pectolytic and Cellulolytic Activities

Pectolytic and cellulolytic activities of the filtered exudate were determined by the viscometric technique of Kelman and Cowling (1965). A 0.25 percent carboxymethyl cellulose (Hercules Inc. CMC-70) solution in 0.05M citrate buffer at pH 5.5 was used as the substrate to determine the cellulase activity. A 0.25 percent sodium pectate solution in 0.05M sodium citrate buffer at pH 5.5 was used to determine pectolytic activity. A Cannon-Manning semi-micro viscometer (numbered 199 A883) with a charge capacity of 0.55 ml was used and all assays were carried out at 30°.

In the viscometric assays, 0.9 ml of the substrate was added to the viscometer and allowed to equilibrate to 30°. Exudate of 0.1 ml was then added and the change in viscosity recorded as a change in running time in seconds. Autoclaved exudate plus substrate was used as control.

iii) β -Glucosidase

β -Glucosidase activity was determined by the method of Cohen et al. (1951). Filtered exudate was used directly; the control was autoclaved exudate plus substrate alone. This method is based on the release of 6-bromo-2-naphthol from 6-bromo-2-naphthol-B-D-glucopyranoside by B-D-glucosidase and the formation of a colour complex with tetrazotized diorthoanisidine. The colour was measured on a spectrophotometer at 540 nm. A standard curve using 6-bromo-2-

naphthol was prepared and the number of moles of substrate transformed was read directly from the curve. The specific activity of the enzyme was calculated.¹

iv) Protease

Protease activity was determined by the procedure of May and Elliot (1968), using 1 percent casein (Hammerstens) in 0.1M Sorensen's buffer at pH 7.6. Incubation of the substrate was carried out at 35° and the reaction terminated by the addition of TCA. The tyrosine and tryptophan released by the action of the enzyme was determined by the absorption of the supernatant solution at 280 nm. A unit of protease activity is defined as that amount of enzyme which produced an increase in absorbance at 280 nm of 0.05 in forty minutes at 35°.

v) Ribonuclease

RNase activity was determined by the method of Wilson (1963). The substrate (Sigma yeast RNA) was dissolved in a 0.125M cacodylic acid buffer at pH 5.0, and the reaction carried out at 37°. The hydrolysis of RNA by RNase releases split products which are not precipitated by the perchloric acid-uranyl acetate solution, and these split products are determined by spectrophotometric analysis of the supernatant at 260 nm. A unit of RNase activity is defined as that amount of enzyme which produces an increase in absorbance at 260 nm of 0.1 in thirty minutes.

¹The specific activity is defined as the number of enzyme units per mg protein where, by international agreement, 1.0 enzyme unit is defined as that amount of enzyme causing transformation of 1.0 micromole of substrate per minute at 25°.

vi) Acid Phosphatase

Acid phosphatase was determined by the method outlined in the Sigma Technical Bulletin No. 104 (1971), revised edition.

Incubation of the substrate p-nitrophenylphosphate in 0.05M citrate buffer at pH 4.8 was carried out at 37°. The compound p-nitrophenylphosphate is colourless, but hydrolysis of the phosphate group by the enzyme liberates p-nitrophenol which produces a colour that can be read directly at 400 nm.

A p-nitrophenol standard curve was prepared and the moles of substrate transformed were determined directly from the curve.

4. Oxalic Acid

Oxalic acid was determined by the method outlined by Beer et al. (1965). A 0.5 ml sample of filtered exudate was added to an twenty cm centrifuge tube and 0.4 ml of CaCl_2 (saturated solution in 5 percent acetic acid) added. The mixture was mixed and allowed to stand overnight. The contents of the tube were centrifuged at $3000 \times g$ for ten minutes and the supernatant discarded. The precipitate was washed three times with cold, 5 percent acetic acid-saturated calcium oxalate solution and centrifuged each time at $3000 \times g$ for ten minutes. The supernatant was discarded each time.

The sediment so obtained was dissolved in 1.0 ml of 4N H_2SO_4 , transferred quantitatively to a 50 ml beaker and heated to 90° on a steam bath. The hot solution was titrated to a faint pink end point with 0.02N KMnO_4 .

$$1\text{ml } 0.02\text{N } \text{KMnO}_4 = 0.9\text{mg anhydrous oxalic acid.}$$

5. Reducing Sugars

The method used was that of Nelson 1944, as modified by Somogyi (1945). The solution to be tested was reacted with alkaline copper in a boiling water bath; then, the reduced copper was determined by the addition of arsenomolybdate chromogenic reagent which produced a blue colour with the reduced copper. Quantitative estimation of the colour was determined spectro-photometrically at 500 nm against a reagent blank.

Glucose was used to prepare a standard curve.

Degradation of Plant Tissue

1. Potato Disc Experiments

The effect of the filtered exudate on potato was determined by estimating a) wet weight loss, b) dry weight loss, c) release of reducing sugars, and d) direct observation of the exudate-treated discs using the scanning electron microscope. Twenty potato discs, 10 mm in diameter and 200 μ thick, were cut from the central portion of potato tubers using a cork borer and a hand microtome. The discs were thoroughly washed in three changes of tap water and the washed discs placed in a 15 cm test tube with 0.5 ml of citrate buffer at pH 5.5. Filtered exudate (0.1 ml) was added and the mixture gently agitated on a Burrell wrist action shaker for various time intervals.

In experiments designed to determine the wet weight loss, the discs were washed three times, blotted on a paper towel to remove excess surface water, and the weight recorded. They were then incubated with the exudate-buffer mixture, for various time intervals (Fig. 37), removed, washed as described, and the weights recorded again.

After the wet weights were taken, the discs were then dried overnight at 95° in a forced draft oven and reweighed.

Both a buffer control and autoclaved exudate were run for comparisons.

Quantitative determination of the reducing sugars released during tissue degradation experiments was carried out by analysis of the supernatant liquid as follows: the buffer solution in which the discs had been incubating was drawn into a 1 ml tuberculin syringe and filtered through a 0.45 u millipore filter. This filtered solution was tested for total reducing sugars by the method described above.

2. Effect of Exudate on Live Tissues

The capacity of harvested exudate to react on live tissue was tested in tomato stems, tomato fruit (both field and greenhouse grown), and in field grown sunflowers. The tomato varieties used were Earliana and North Star. The variety of sunflower used was Giant Russian. Tomato stem injections were made on young seedlings approximately 30-40 cm in height, while the age of the fruit used varied and was designated as ripe (red) or unripened (green). The sunflowers used, were at the stage just prior to the heads opening.

A Stylet 1 cc tuberculin syringe with a #25/ 5/8 needle was used. The tomato stems were injected centrally into the pith, in the central region of the node above the lower leaves, by angling the needle into the stem. Tomato fruits were injected into the blossom end, about 5 mm off centre and the needle was inserted approximately 1 cm into the core of the fruit.

Injections into the sunflower head were made into the pith area from the underside of the head and several injections were made into each head.

The results were recorded at various time intervals by photographing the affected tissue.

Thin Layer Chromatography

Samples were prepared for thin layer chromatography by ultrafiltration on a Millipore 13 mm, stirred, ultrafiltration cell under a nitrogen pressure of 60 psi. Pellicon membrane filters PSJM, PSED, and PSAC were used. They represent molecular weight cut-offs of 100,000, 25,000 and 1,000 respectively. The 1,000 membrane, PSAC, gave sufficient clean-up for clean TLC runs. The exudate was collected in the normal manner (for potato-disc experiments, the supernatant fluid was withdrawn by syringe), and in each case, added to the filtration cell and run through the three filter sizes in sequence. Filtration required approximately one hour for each run.

The filtered material was immediately spotted on 20 x 20 cm plates and coated with silica gel G in 0.1 M boric acid (50% W/V). The silica gel was coated on the plates to a thickness of 0.25 mm using a Quickfit automatic plate leveler with an adjustable spreader. The plates were dried overnight at room temperature.

Two solvent systems were used: 1) n-propanol-ethyl acetate-water 7:1:2, 2) acetone-formic acid-ethanol (95%) 3:1:1. The first solvent system was run 4 1/2 hours and the second 1 1/2 hours. Development was carried out in glass tanks, presaturated with the solvent system in use.

Detection of the sugars was carried out with the following stain: 4 percent ethanol-aniline, 4 percent ethanolic diphenylamine, 85 percent phosphoric acid - 5:5:1. After spraying, development of the colour was accomplished by drying the plates at 120° for twenty to thirty minutes.

Scanning Electron Microscopy

The breakdown of potato tissue by exudate solution suggested the possible destruction of the cell wall of the individual cells of the potato discs used. The procedure used for examining the cell wall surface was, as follows. The discs were washed as previously described. They were then immersed in 3 percent glutaraldehyde in phosphate buffer, 0.02 M, pH 7.4, for three hours at room temperature. The specimens were then washed with three successive washings of tap water, placed in 3 percent osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, overnight. After three, thirty minute rinses with tap water, the discs were dehydrated as follows. Equal amounts of tap water and absolute ethanol (99.8%) were added to the specimen vials. For the next three changes of alcohol, 3/4 of the liquid in the vial was removed and replaced by absolute ethanol. The final dehydration was in absolute ethanol. The discs were kept in each alcohol solution for thirty minutes.

The discs were then placed in 75 percent amyl acetate in absolute ethanol and left overnight.

For scanning, the discs were critical-point dried using liquid carbon dioxide in a DCP-1 critical-point drying apparatus (Denton Vacuum Inc.), attached to an aluminum stub with silver dag, and the

disc coated with carbon and then gold on a rotary stage in an Edwards vacuum coating unit, Model E12E. Total thickness of the coating was about 250 Å. The discs were examined with a Cambridge stereoscan scanning electron microscope S4 at 20K volts.

CHAPTER III

RESULTS AND DISCUSSION

Exudation

Maturing colonies of Fusarium culmorum exhibit three distinct growth zones which are designated as A, B, C, in Figure 5b. Zone A, located closest to the point of inoculum, is the major region of spore production and the hyphae of this zone vary in morphology (Fig. 6). Some of these are older hyphae, which are associated with surface growth; they are thick-walled and highly septate, and in their growth, tend to radiate out toward the colony front with only a few lateral branches. They can be followed for considerable distances without major interruption when observed microscopically. These thick hyphae are sometimes highly pigmented with the red pigment rubofusarin (Ashley et al., 1937) which characteristically stains the agar medium a deep red in this species.

Another type of hyphae in this zone are thin strands with relatively few septa and no pigmentation. These hyphal strands tend to be aerial, are highly branched, and grow in all directions forming an interconnected mat of mycelium (Fig. 6) rather than a radiating pattern.

The third type are rhizomorph-like strands of hyphae (Fig. 6) which are held together by an undefined material, which will be discussed in more detail later.

Macroconidia are abundant in this zone while microconidia are absent. The macroconidia are produced from the phialides on conidiophores which are initially generated on aerial mycelium, and eventually large, black sporodochia are formed. These findings are as those described by Booth (1971) for the sporulation pattern of Fusarium culmorum (W. G. Smith) Sacc.

Zone B (Fig. 5b) is a transition zone between zones A and C. This zone is also pigmented but with the gold pigment aurofusarin, which is closely related chemically to the rubofusarin found in zone A (Ashley et al., 1937). As the colony continues to age, this gold pigment gives way to a deep red colouration in the colony, and the change is likely related to a change in the chemical environment within the staling medium and hyphae. This zone also contains a variety of hyphae contained within zone A, but with relatively more of the thin aerial type being present. There is considerably less sporulation in this zone but the macrospores are generated in the same manner as described above, although there are no sporodochia present.

Zone C is the most active and uniform zone in relation to hyphal extension. Being the front of the colony, it is associated closely with active growth, cell wall synthesis, branching, and nuclear division. This is the region of droplet formation. It consists mainly of thin, uniform, aerial mycelium, but also has considerable surface and submerged growth at the very periphery of the zone. There is no pigmentation evident here.

With the particular experimental conditions used for determination of droplet distribution, zones B and C always appear relatively narrow in comparison to zone A. The linear ratio is: zone A:B:C =

5:1:1 approximately, whereas by area, the ratios would be approximately 2.5:1:1. By visual observation of the colony growth, it can be concluded that these ratios remain relatively constant throughout the constant growth phase (48 to 96 hours).

After inoculation, the following events occur. For some time (approximately 24 hours), the colony consists of only a typical non-pigmented C zone which contains very few thick hyphae and no rhizomorph-like strands. This could be considered the lag phase or establishment period for the inoculum and is actually a colony consisting of only C zone. Sometime, between 24 hours and 48 hours, pigmentation occurs around the inoculum and then, as the colony develops, zones A and B develop. At this stage, a developing colony consists of three zones: a new C zone constantly being regenerated at the periphery as hyphal extension occur; a B zone, which is transitional and shows early signs of aging such as pigmentation and hyphal differentiation; and an A zone in which aging is advanced and masses of spores and sporodochia formation are obvious.

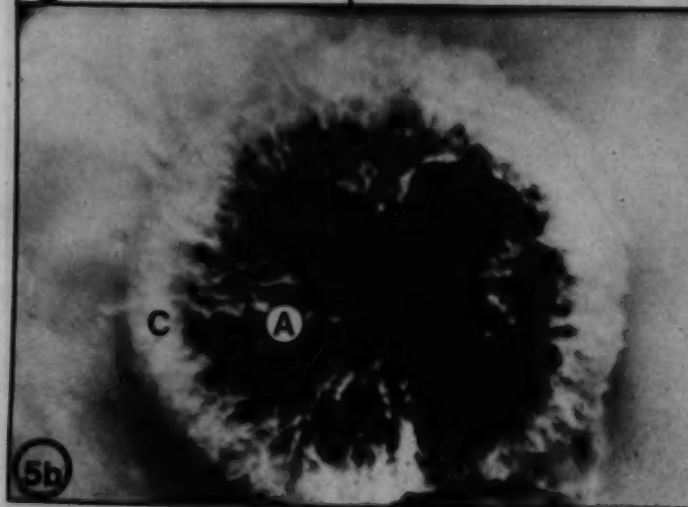
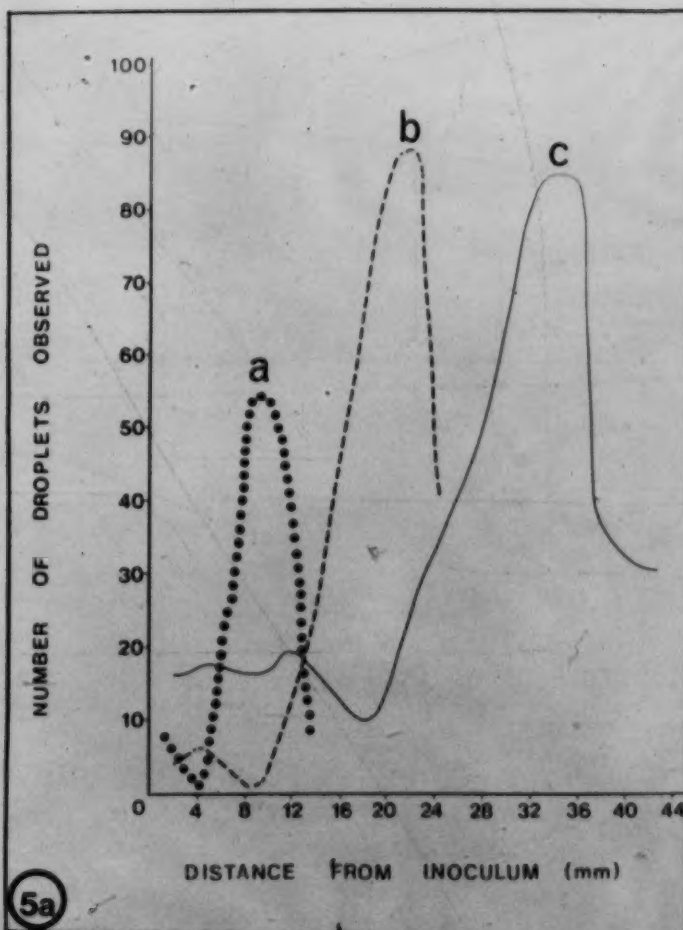
Figure 5a shows that droplet formation and distribution are interrelated to colony growth. At 48 hours, the peak droplet distribution occurs 8-10 mm from the point of inoculation; at 72 hours, this peak has shifted with the droplet concentration, now highest at 19-21 mm from the point of inoculation; and at 92 hours, has shifted again, with the droplets now concentrated at the 30-34 mm point. In each case, the zone containing the droplets, represented by peaks on the graph (Fig. 5a), disappears in time; and the area of the colony represented by that peak becomes devoid of droplets and transformed into a more advanced state of maturation (Figs. 8a-d). The fate of the drop-

FIGURE 5a

The number of droplets at various distances from the point of inoculation with E. culmorum. Counts were made at 48, 72, and 92 hours and the peaks on the graph indicate a concentration of droplets at the colony periphery at these respective times. Curve a = 48 hours; b = 72 hours; c = 92 hours.

FIGURE 5b

A colony of E. culmorum illustrating the droplet zone "C" and the two pigmented zones "A" and "B".



lets as maturation advances is explained in more detail below.

Droplet Characteristics

Aerial droplets in a given region may exist in various stages of development. Initially, they are generated at or near the hyphal tip (Fig. 7), and at this stage, they are watery in appearance and completely hyaline. Observation shows that they may appear in groups within a specific area of the colony or in bead-like fashion on a single hyphal strand (Fig. 8). These are not swellings on the hyphal wall as they seem to appear, but rather liquid drops resulting from tip growth continuing as droplet formation continues to occur.

Observation of surface growth shows that the growing hyphae in contact with the surface are surrounded by liquid (Fig. 9). A scanning micrograph of hyphae grown on a cellulose membrane (Fig. 10) shows a residue associated with the hyphal strands left behind after the hyphal-liquid is dehydrated. This can be interpreted as liquid generated by the same mechanism, but dispersed by surface tension as a result of physical contact of the hyphae with the solid agar surface. Solubilization of the agar occurs from the interaction of the agar with fungal exudate. A scanning micrograph (Fig. 11) shows surface hyphae of *E. culmorum* produce distinct channels in the agar which are about twice the diameter of the hyphae. Presumably, under these growing conditions, the hyphae are generating liquid which is hydrolytic and which enables them to penetrate the substrate. Both these examples give evidence to support the secretion principle.

One of the more noticeable characteristics of droplets in early stages of formation is that liquid is drawn back into a hypha

FIGURE 6

E. culmorum surface mycelia in zone "A" showing the variety of hyphal types in this zone. T = thicker hyphae (note distinct septa). t = thinner hyphae in the same area. R = rhizomorph-like strands characteristic of the "A" zone.

FIGURE 7

A single transparent droplet surrounding the hypha at the tip on a colony of *E. culmorum*.

FIGURE 8

Time sequence over 140 hours of one area of a colony of *E. culmorum*. (a) An area within the "A" zone approximately 48 hours after inoculation. At this time the droplets (C) have accumulated in a group and are still in the early transparent stage. (b) Droplets have become granular (G) at 96 hours. (c) Hyphae are grouped together and macroconidia (S) are visible (120 hours). (d) A more advanced state of development showing large numbers of conidia. Note: the arrows in 8c and 8d show conidia have formed where a droplet had been just a few hours before.

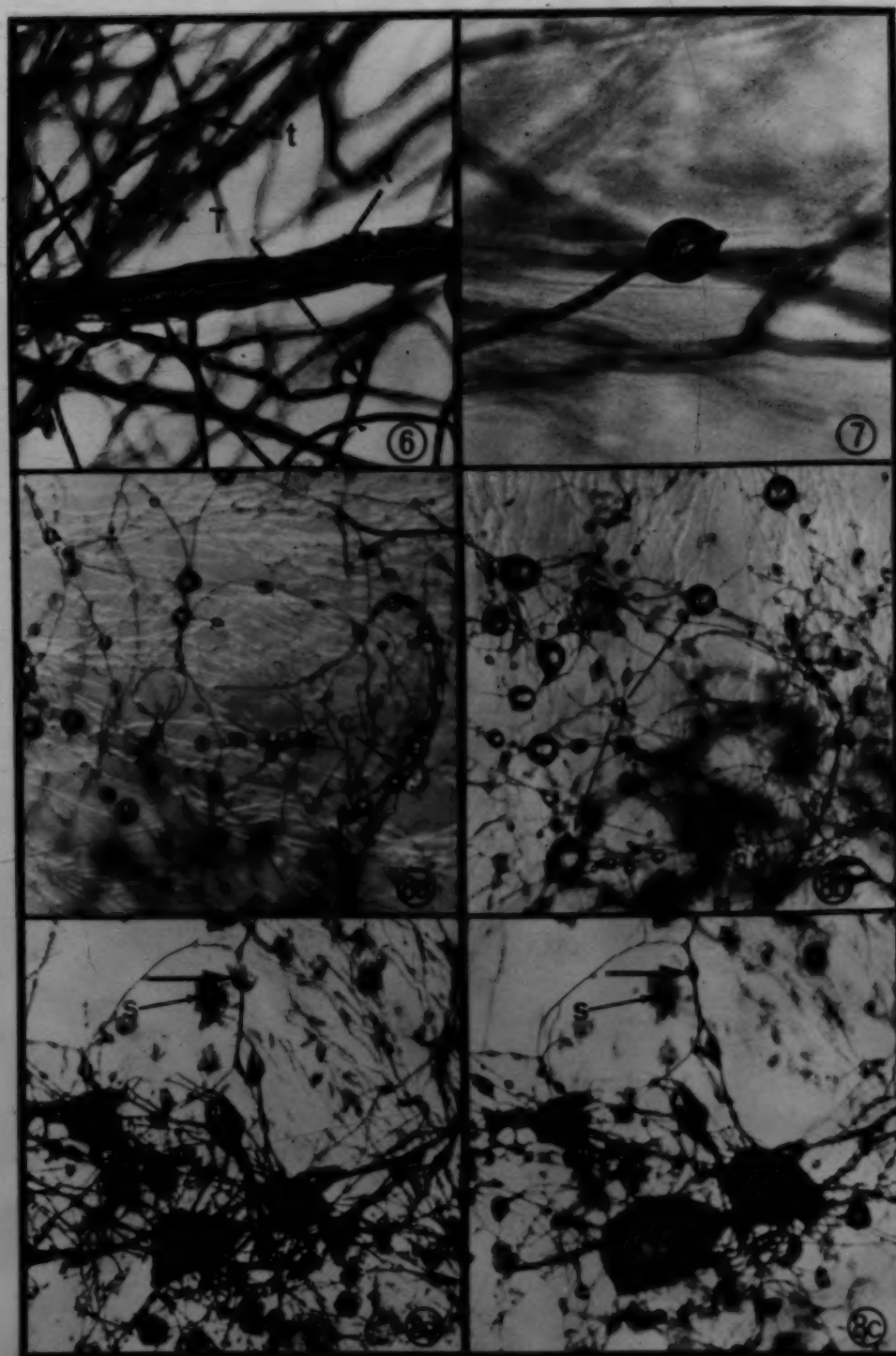


FIGURE 9

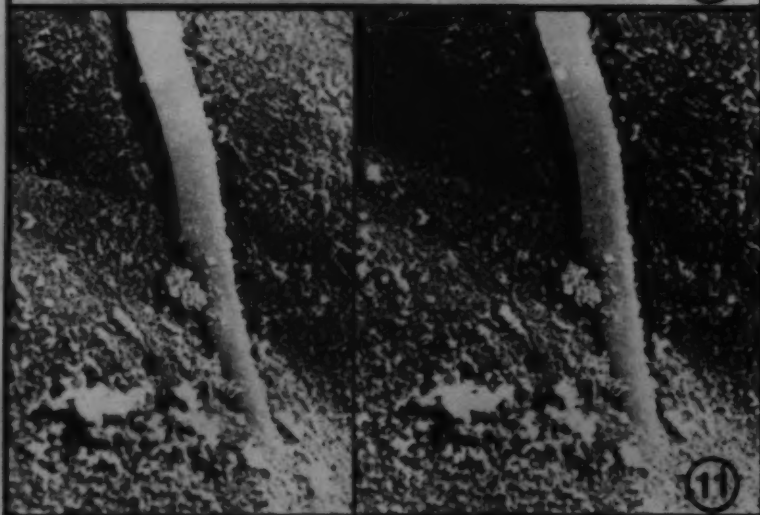
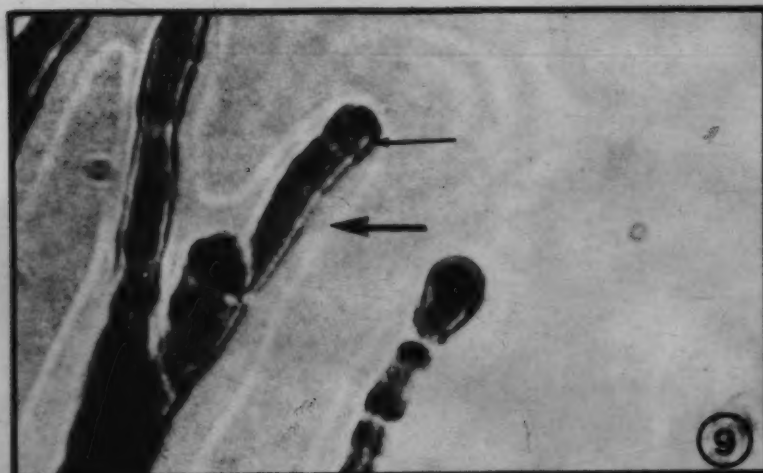
A surface photo of the hyphal tip area of a 6-7 day old E. culmorum colony showing liquid surrounding the hyphae in contact with the surface (large arrow). The small arrow indicates the hypha.

FIGURE 10

A scanning electron micrograph of a hyphal tip of E. culmorum showing a residue surrounding the tip (arrow).

FIGURE 11

A E. culmorum hyphal strand growing out of the PDA agar surface. These two pictures are a stereo-pair, and when observed through stereo glasses the area indicated by the arrow shows a trench in the agar under the hyphal strand.



and then re-exuded (Figs. 12a-c). In this sequence, it is removal of the petri plate lid which allows reappearance of the droplet, within seconds, at precisely the same point on the hypha. Relative humidity is probably the controlling factor, although excessively high moisture is not a prerequisite. Growing colonies under normal room humidity produce numerous droplets; and those associated with the older portions of the colony tend to dry after removal of the petri plate lid, leaving a granular residue on the surface of the hyphae. The phenomenon of reabsorption and re-exudation occurs only with very young droplets and near the hyphal tip. The mechanism by which this occurs is unknown.

Frequently, hyphal strands in the presence of droplets may be drawn together (Figs. 6, 14) to produce thick, multi-hyphal strands characteristic of mature areas of the colony. The force required may be supplied by surface tension between the droplets and the hyphae. Figures 13a-c show this process occurring between two strands, and once the attraction is initiated, the strands come together very rapidly. The material binding the strands together is presumably the mucilaginous residue remaining during and after the water is dissipated. When this occurs in the actively growing C zone, very little residue remains after the water is eliminated. In more mature areas of the colony, where the liquid involved is more viscous and opaque, many strands become involved in this fusion phenomenon. Washing the strands with lactophenol cotton blue shows that these thickened strands are a conglomerate of many hyphae (Fig. 6). This type of hyphal aggregation was described as early as 1889 by DeBary.

FIGURE 12

Absorption and re-exudation of a droplet on a colony of F. culmorum. (a) A droplet in the transparent stage. (b) 30 seconds after removal of the petri dish lid the droplet has been reabsorbed. (c) 45 seconds after removal of the lid. (d) 75 seconds after the lid was replaced the liquid has been re-exuded.

FIGURE 13

This sequence shows the fusion of hyphal strands on a colony of F. culmorum. Arrow 1 (13a) and arrow 2 (13b) show fusion at one point while arrow 2 (13b) and arrow 2 (13c) show fusion at a second point.

FIGURE 14

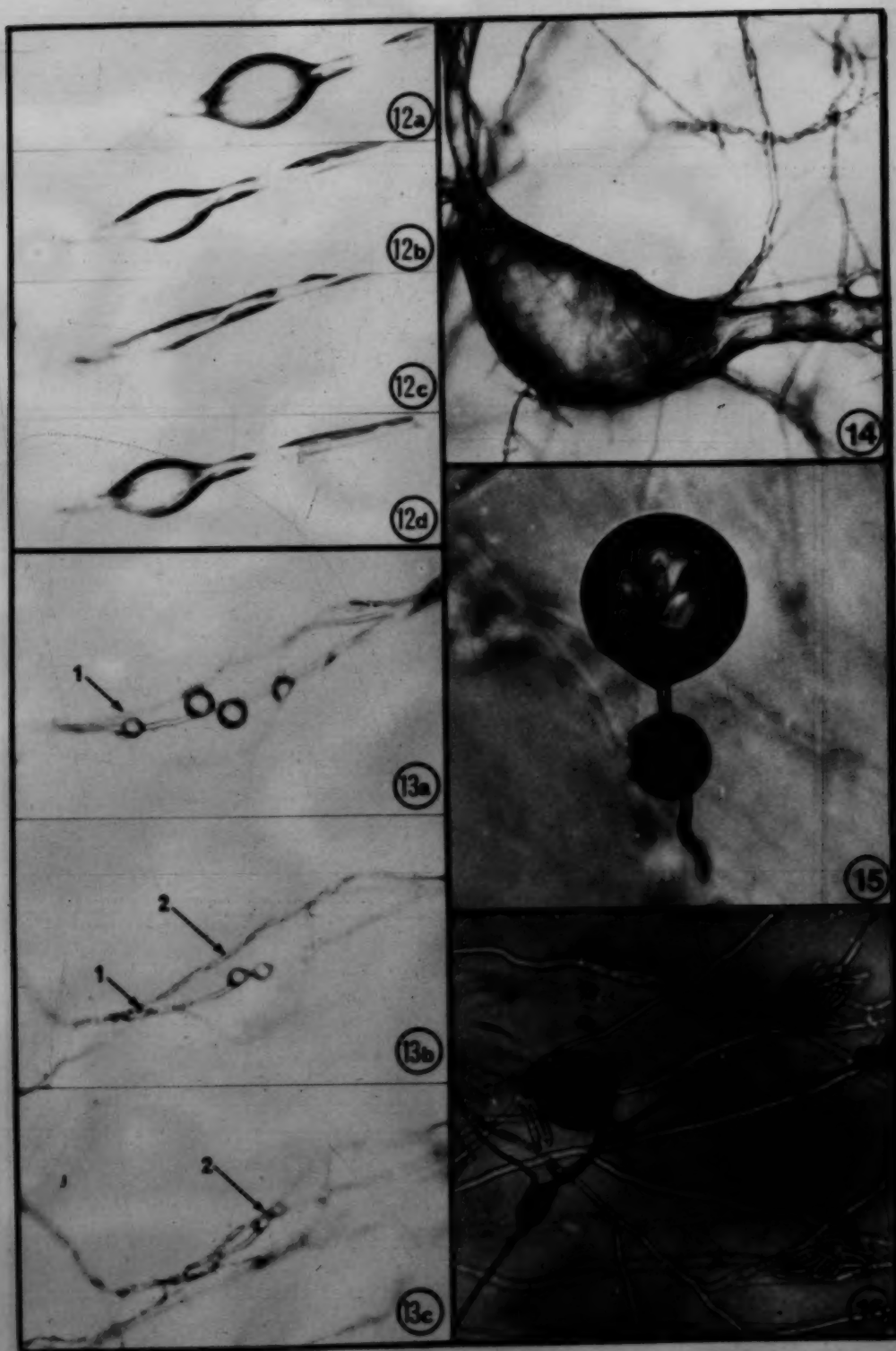
Hyphal grouping on a colony of F. culmorum and the distinctly opaque liquid associated with it.

FIGURE 15

F. culmorum macroconidia within a liquid drop.

FIGURE 16

A group of F. culmorum conidia released from a droplet which has touched the agar surface (arrow). An intact droplet can be seen above the agar surface.



Spore Formation

It is common to observe macroconidia within these hyphal droplets behind the leading edge of the colony (in zone A) during the maturation stages of colony development (Fig. 15). In experiments using 1/10 or 1/100 nutrient concentrations, the association of macroconidia formation was easily correlated with droplet formation. Observation of the growing colony indicated specific areas of heavy droplet formation and other areas with equal hyphal growth, but no droplets. A time study showed that macroconidia production occurred in the areas of heavy droplet formation (Figs. 8a-c). Further observation has shown that the production of macroconidia is always preceded by the presence of liquid drops in the areas of the spore masses, at some time prior to spore production.

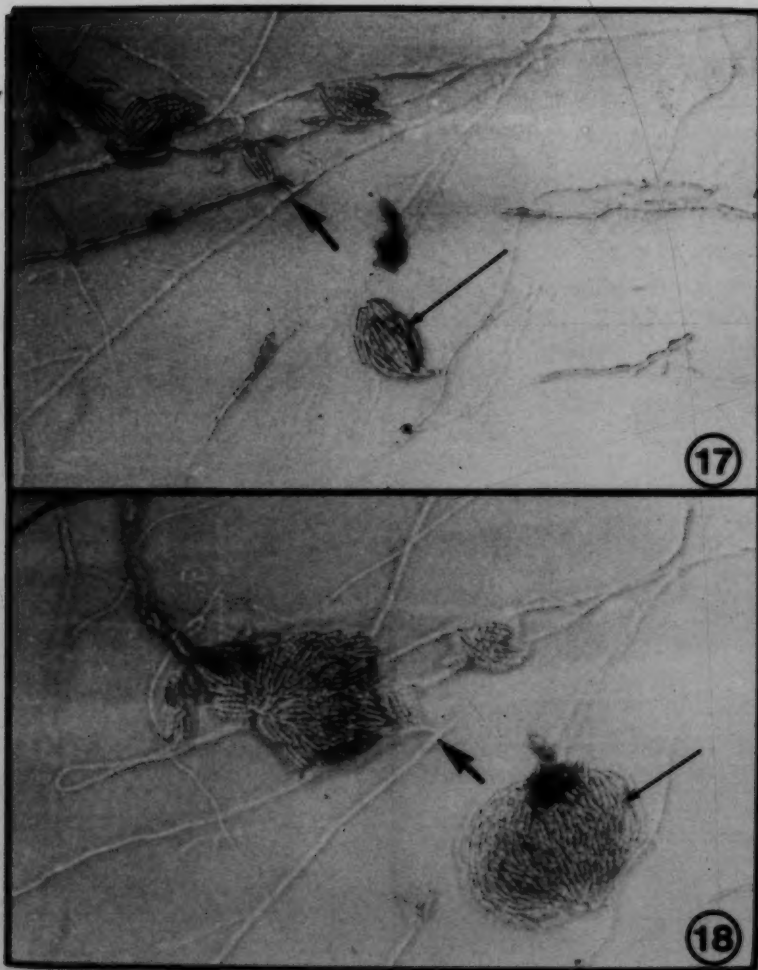
The formation of droplets at the stage shown in Figure 15 (on PDA) is the earliest that macrospores are detected and as these spores continue to accumulate within the droplet, the droplet may be weighed down and the liquid dispersed on the agar surface (Fig. 16). Although the continuity of the droplet is lost when this happens, the influence of the liquid remains and production of macroconidia in the region continues (Figs. 17, 18). The association of conidia production with the liquid, suggests that a chemical and/or physical stimulus is necessary for normal spore production and is supplied by the droplet and its contents. The chemical nature of the stimulus is not known, but it appears to become active during the time that pigmentation is initiated. Older, pigmented agar with most of the mycelium removed, was extracted with distilled water; the extract, after filter sterilization, was spotted on the surface of newly inoculated colonies. As

FIGURE 17

A surface view of a *E. culmorum* colony growing on 1/100 diluted medium and showing a mass of macroconidia (small arrow). The large arrow is a point of reference.

FIGURE 18

Same view as in Figure 17 but 24 hours later. Note the increased number of spores in the mass of macroconidia.



the mycelium grew over these spots, the colony, at that point, became pigmented and spores formed (in other words, the area became characteristic of a B zone and progressed into the A zone state from this). The surrounding mycelia outside the influence of the drops, but of the same age, i.e. equidistance from the point of inoculation, showed no signs of premature aging but rather, aged normally. When this "uninfluenced" mycelium began the transition into a B zone state, the mycelial areas influenced by the drops were well into the A zone state and already had mature sporodochia present. The final result was a normal overall colony with isolated areas of very advanced aging, where the drops of extract had been deposited. Distilled water drops on the colony had no effect on aging.

The sequence of development of a cluster of droplets over a five day period is shown in Figures 8a-d, and the same Figures show the time sequence of spore formation within the same region. Stage "a" is the early transparent stage with no spores present, "b" shows granularization, evidenced by the opaqueness of the drops and strand formation, while "c" and "d" show sporulation. The spores can be seen on the surface of the agar.

In older colonies, the mycelial mat is very thick due to secondary growth, and pools of liquid rather than single drops are formed. These pools are covered with a "sac" similar to that described by Colotelo (1971) on the surface of sclerotial exudate from Sclerotinia sclerotiorum. The sac appears, in this case, to be thinner than that shown by Colotelo, but its presence does indicate that there is some material within the droplets capable of forming a surface film.

These pools or surface drops which are associated with older colonies, form beneath a thin network of hyphae (Fig. 19) and appear to be related to sporodochia formation. A cross section through a sporodochium (Fig. 20), can be directly compared with the surface drop on the surface of the colony, and arrow "a" in Figure 20 shows the top of a mature sporodochium. Arrow "b" (Fig. 19b) illustrates the network of hyphae which characteristically cover these drops and appear to "anchor" them to the agar surface "much like a rope net would anchor a gas balloon to the ground". Arrow "b" (Fig. 20) shows that this covering network of hyphae remains at the advanced stages of sporodochium development and indicates that these are two different stages of the same process. Figure 21 is an enlargement of the interior of the sporodochium shown in Figure 20 and illustrates that the mature sporodochium consists of a mass of heavily pigmented macrospores. Figures 19, 20 and 22 illustrate the stages from drop formation to a mature sporodochium; Figure 19 shows a very early stage with only a few spores present, while Figure 22 shows discrete concentrations of spores deep within a drop. As maturation continues, the drop changes from clear and colourless through shades of amber to a reddish-black; the colour change is related to the number and stage of development of the pigmented macrospores present in the drop. With increasing age, the liquid disappears, leaving a granular mass (Fig. 23). Flooding the colony with glycerol loosens these masses and releases the individual spores (Fig. 24).

So far, these observations reported on the maturation of a colony of F. culmorum on PDA and the related phenomenon of hyphal exudation suggest a close relationship between the two. The detailed

FIGURE 19

(a) Top view of a large drop amongst the secondary hyphae of an older colony of E. culmorum. Note the macroconidia within the liquid near the surface of the drop. (b) Side view of the same droplet (arrows "a" and "b" indicate areas of the droplet for comparison with Figure 20).

FIGURE 20

Transverse section of a sporodochium on E. culmorum. Note the structural similarity between the sporodochium and the drop in Figure 19b. a = top of the drop; b = hyphal network.

FIGURE 21

An enlargement of Figure 20 showing macroconidia on E. culmorum.

FIGURE 22

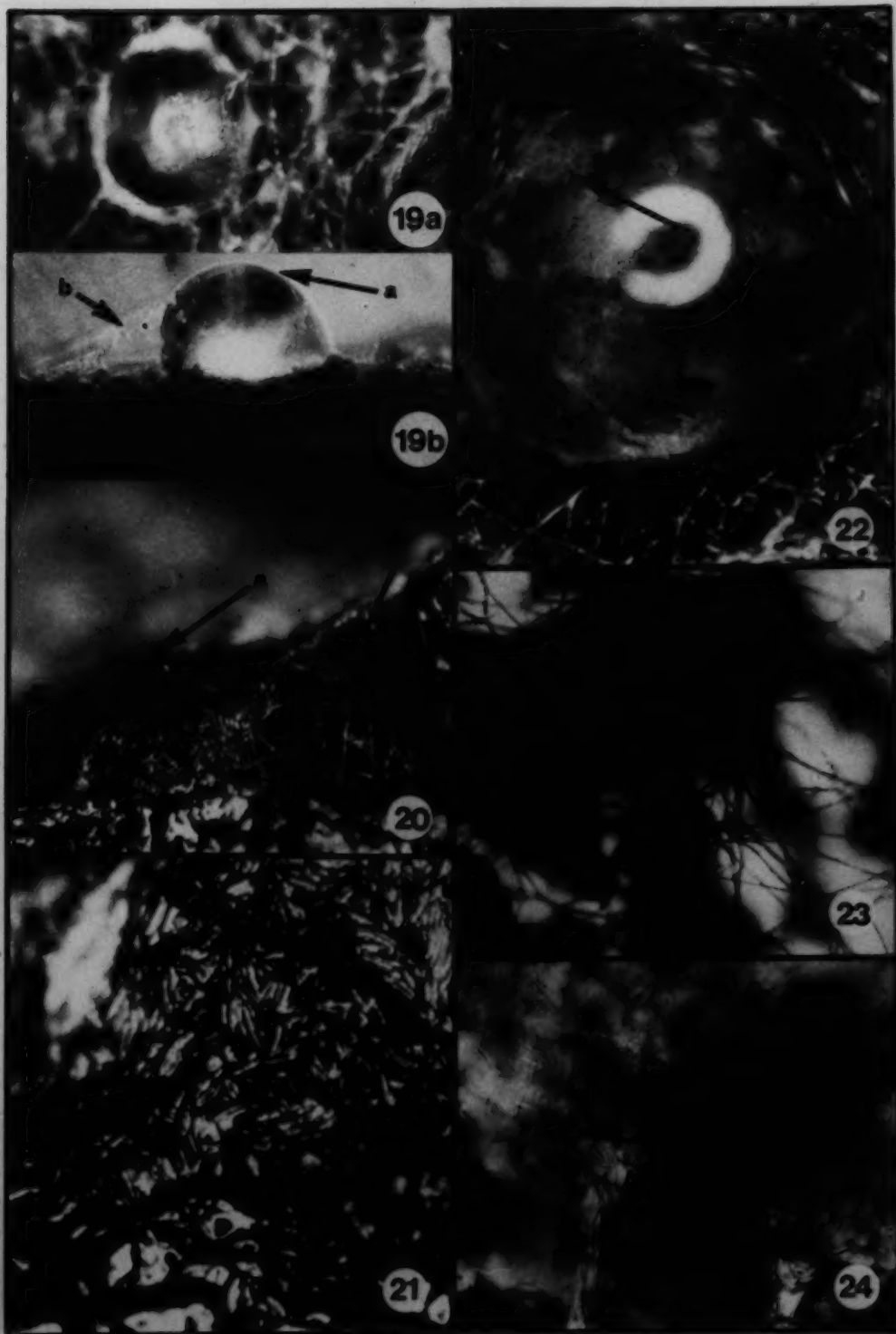
Another stage in macroconidia formation on E. culmorum. Note the large mass of macroconidia contained within the drop.

FIGURE 23

The black shadowy images represent masses of conidia on the agar surface of a colony of E. culmorum which appear as a granular mass.

FIGURE 24

The granular mass on the E. culmorum colony shown in Figure 23 was flooded with glycerol and the individual macroconidia released.



description presented here for F. culmorum represents what is a general occurrence of exudates on the growing hyphae of numerous fungi. During the preparation of this research, exudates have been observed, associated with the development of cultures of F. culmorum, F. caeruleum, F. sambucinum, Sclerotinia sclerotiorum, Sclerotium rolfsii, Pythium ultimum, as well as F. culmorum growing on potato and tomato, S. sclerotiorum on sunflower, tomato and carrot.

The general occurrences of these exudates, their apparent influence in sporulation in the case of F. culmorum, and the fact that not only are there solid materials remaining after removal of the liquid, but also, the presence of the droplets in cases of parasitism, all naturally lead to an investigation of the biochemical composition of the liquid being exuded from the hyphae, for the purpose of relating the contents to the physiology of pathogenesis.

The Biochemical Complexity of the Exudate

For collection of the exudate, the fungus was inoculated and grown on dialysing membranes as described under Material and Methods. This resulted in a growth sequence slightly different than previously described; the advantage to this was stimulation of more aerial growth and delayed pigment formation, which broadened the "C" zone, to the extent that after twenty-four hours, it was about 1 cm in radius with no pigmentation visible.

Removal of mycelium for the purpose of collecting exudate for a twenty-four hour analysis invariably resulted in the collection of much surface liquid. It was assumed that this liquid was identical to that associated with the aerial hyphae, with the exception that it

had been in contact with the substrate. For forty hour collections, the colony consisted of mainly aerial mycelium which could be collected without removal of any surface mycelium. At this stage, pigmentation is just beginning to show and the entire "C" zone is easily harvested with no visual disturbance of the surface mat. From sixty hours and up, colonies were pigmented and secondary growth was present.

Protein Determinations

As is characteristic of biological systems, a large number of proteins might be expected in the exudate, especially if enzyme activity is suspected, and since significant amounts of residual protein are found in plant cell walls. The presence of protein as an integral part of fungal cell walls is less well established, although it has been detected (Aronson and Machlis, 1959; Bartnicki-Garcia and Nickerson, 1962; Crook and Johnston, 1962); but, in relation to enzyme protein, structural cell wall protein would be a minor component of the exudate.

A total protein determination was carried out on all exudate runs gathered at various colony ages for use in enzyme assays, and for gel electrophoresis analyses. Figure 25 gives a plot of the average values (four separate determinations done in triplicate) and illustrates the consistent profile for protein content in the exudate collected from these colonies. Much of the variation shown in the standard deviation bars represents variation between runs rather than variation within runs. For example: the low initial value at twenty hours, the increase in protein concentration to a maximum at sixty to ninety hours, and a decrease after ninety hours represents a general rule. The large standard deviation at ninety hours (upper curve, Fig.

25) results from a low value of 14, and a high value of 25.6 mg/ml, but Figure 26 shows that the pattern for each of these runs is characteristic of the average profile shown in Figure 25. (The results for all runs are recorded individually in Appendix A.)

Variation between runs may be explained as variations in the medium, the inoculum, and in the source of the membrane material used. The time interval between inoculation and initiation of linear growth, for example, may vary enormously depending on the type of inoculum or nature of the medium (Mandels, 1955; Page, 1961). There appear to be two groupings for total protein, one with a higher maximum than the other (Appendix A, page). The curves in Figure 26 are representative of the two groups. The abrupt shift to generally higher protein values for all ages of exudate coincides with a change in media from PDA-D to PDA (see Materials and Methods for explanation). Variations were also noticed when the dialysing membrane supplier was changed. Here, the explanation may lie in the fact that there are differences in the membrane thickness.

The total protein in mycelial extracts is relatively constant for the differently aged colonies from which they were taken (Fig. 27). This suggests a rather constant enzyme pool within the cellular cytoplasm of an actively growing and maturing colony. Figures 28a, b and c show the protein profiles on acrylamide gels for both exudate and mycelial extract. (Extracts were made of the same mycelium used for exudate collection and duplicate runs were carried out to confirm the results reported.)

There are a number of protein bands present in the gels and the pattern resulting from each colony/age differs, indicating a dynamic

FIGURE 25

Protein content of the exudate from *F. culmorum* colonies at various colony ages. The curves shown represent a general trend and the large standard deviation is discussed in the text. The lower curve represents a plot of the mean values for exudate from hyphae grown on PDA-D. The upper curve represents a plot of the mean values for exudate from mycelium grown on PDA. (Standard deviation bars are shown).

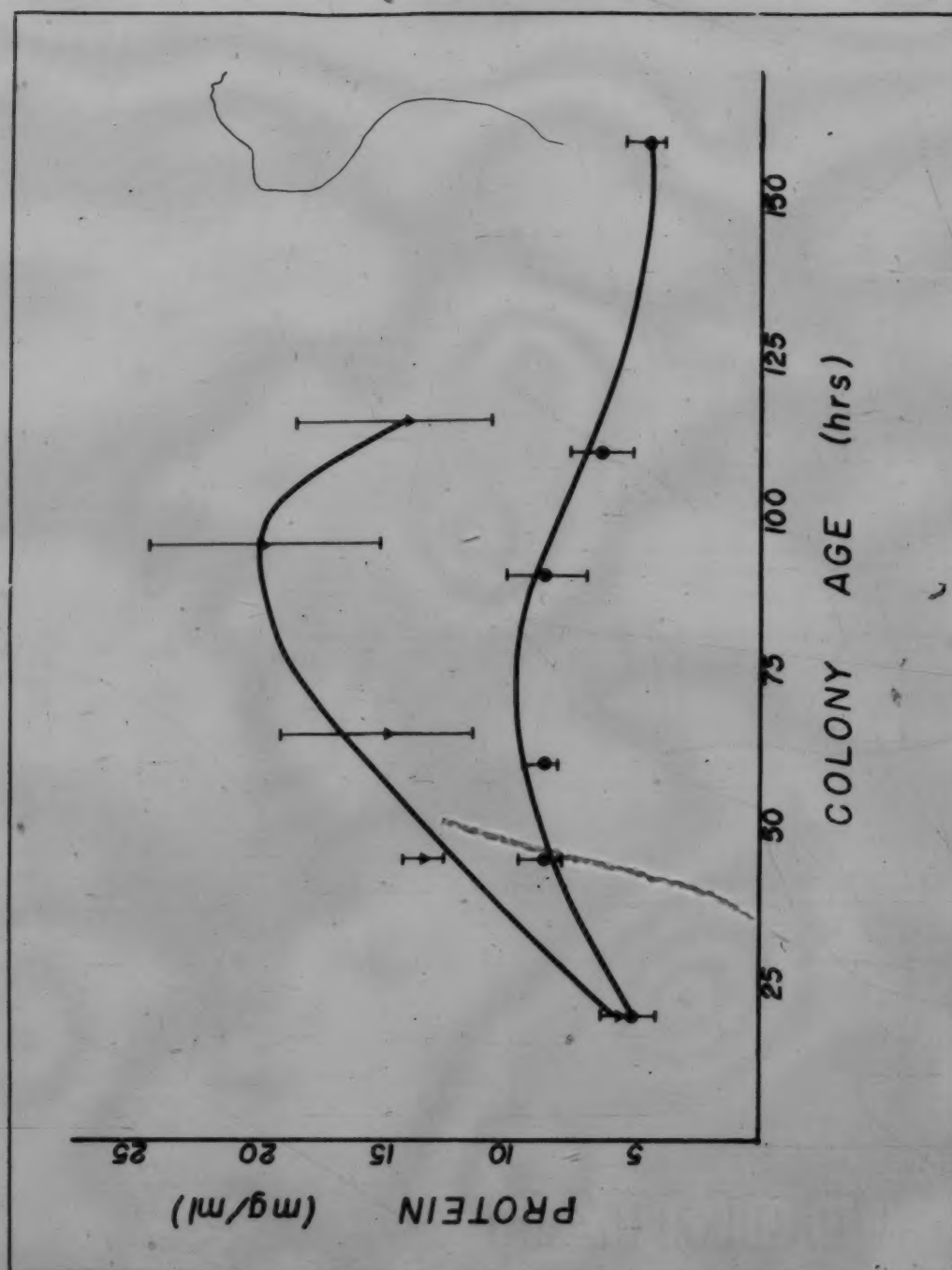


FIGURE 26

Protein content of the exudate from mycelium of
F. culmorum as determined by the Lowry method. Two
independent runs are shown.

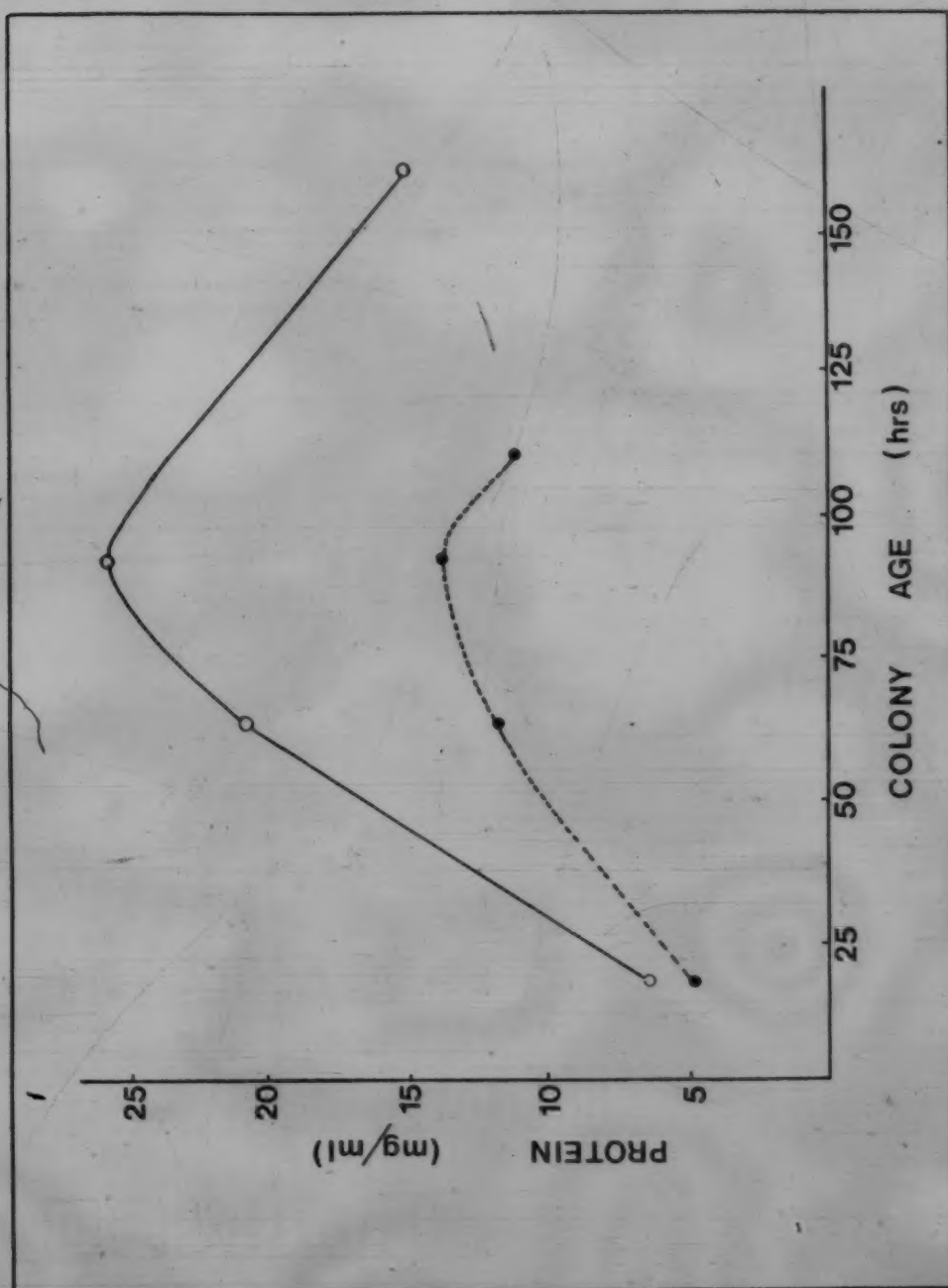


FIGURE 27

Protein content of extract from the mycelium of E. culmorum
colonies of various ages.

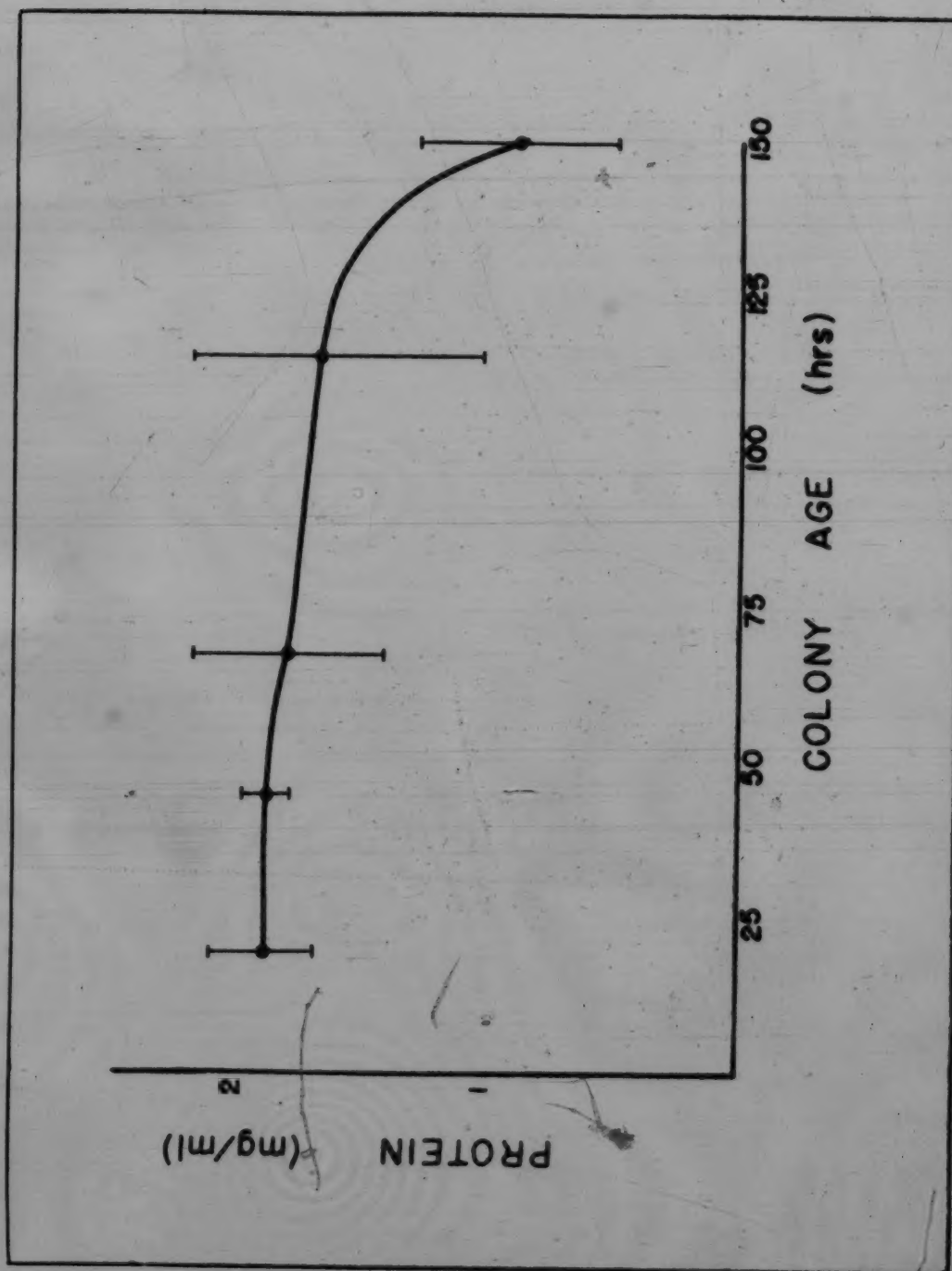
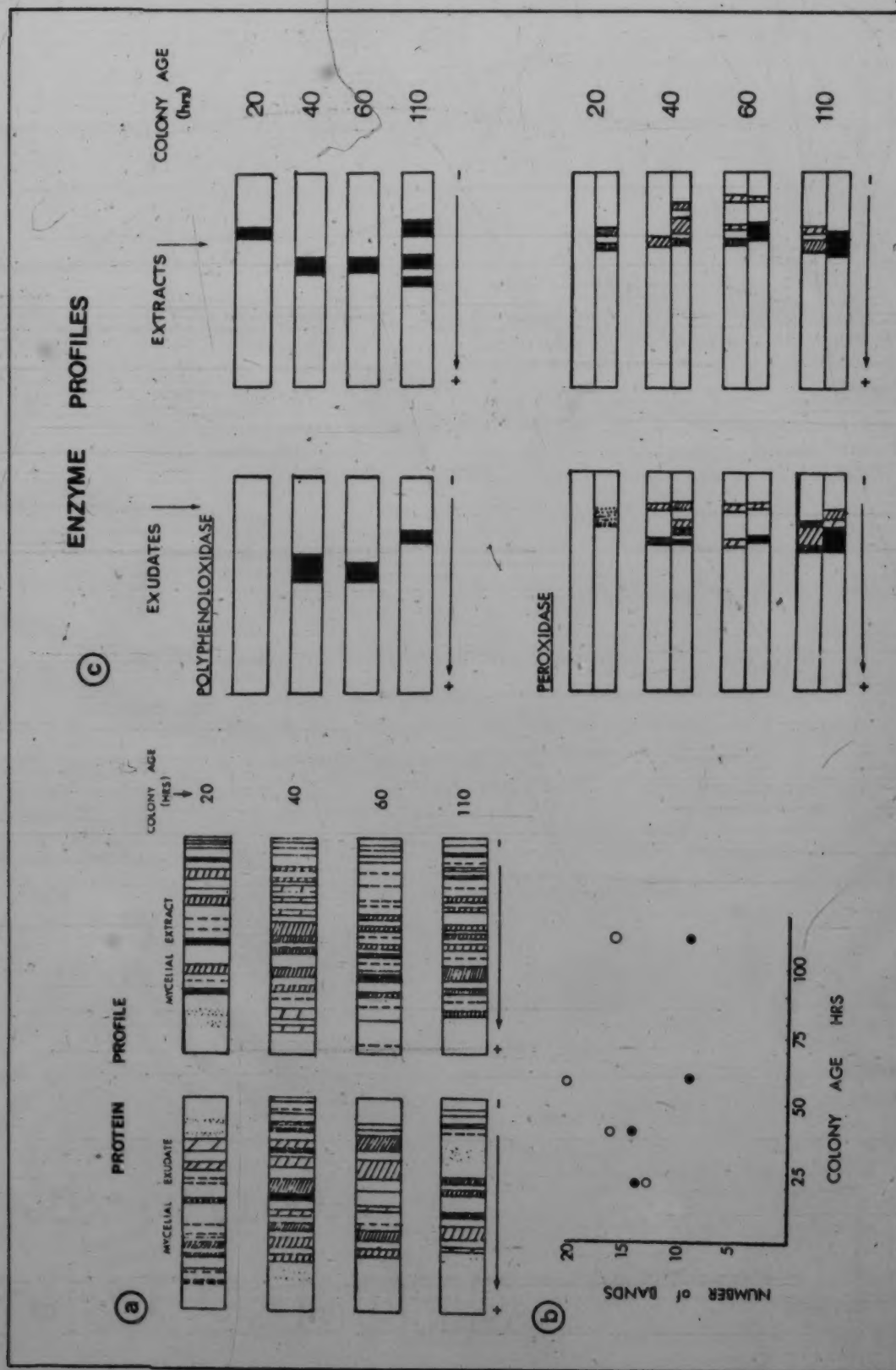


FIGURE 28

(a) Protein profiles on acrylamide gels for exudate and for extract of the mycelium from which the exudate was collected. Four different aged colonies were used.

(b) A plot of the numbers of protein present at each stage in (a); o mycelial extract, ● exudate.

(c) Qualitative peroxidase and polyphenol oxidase determinations by acrylamide gel electrophoresis for both exudate and extracts of the mycelium from which the exudate was collected. The peroxidase gels show two strips. The upper was stained with benzidine hydrochloride, and the lower with pyrogallol.



aspect to the contents of the exudate. Of most significance are the differences between the exudate profile and the extract profile. A comparison of the two shows both qualitative and quantitative differences. Figure 28b illustrates a decrease in the number of bands associated with the exudate at sixty hours, a time when the colony has almost overgrown the plate. This is also the time when staling products have begun to build up at the colony front and on a normal growth curve, would represent a negative growth acceleration phase.

When electrophoresis patterns are obtained from colonies over 110 hours old, a decrease in the number of bands is observed, even for the hyphal extracts, which indicates an extreme deterioration of the system.

Some acrylamide gels were stained specifically for polyphenol oxidase and peroxidase (Fig. 28) and the major conclusions from these tests are that there are enzymes present in the exudate and that they are not common respiratory enzymes, but enzymes which may be associated with tissue degradation. The polyphenol oxidase and peroxidase enzymes may be involved in electron transfer, although this has not been shown conclusively for fungi. Despite their function within the fungus, the presence of these components within the exudate offers possibilities for interaction with host phenolics.

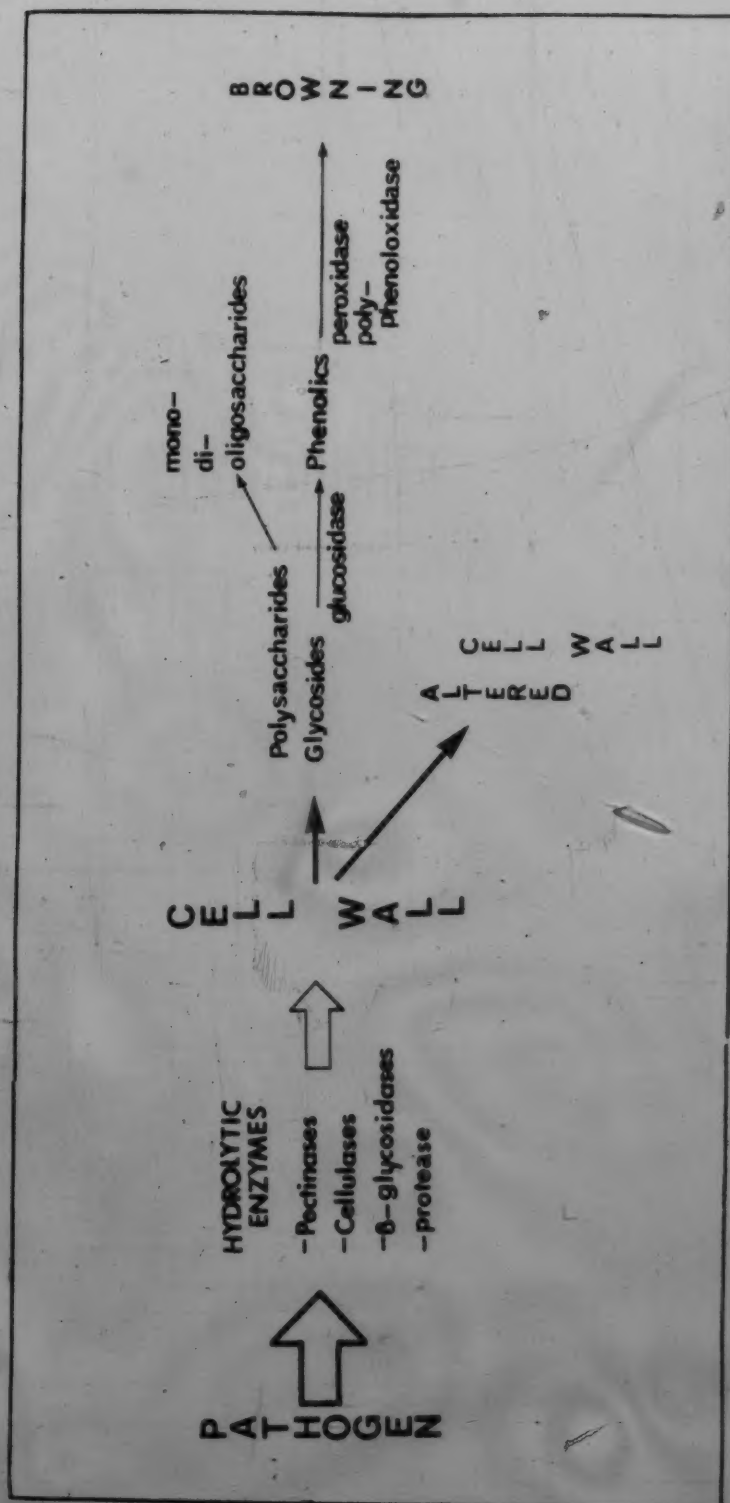
Quantitative Enzyme Assays

The hypothesis that has been made is that the hyphal exudates are of physiological importance and may have influence in the process of pathogenesis. To confirm this latter point, two things were considered:

- i) which specific enzymes are present in the exudate that may aid in

FIGURE 29

A schematic equation for the breakdown of cell walls by a fungal pathogen.



establishment of the pathogen;

ii) does the exudate have any in vivo effect on host tissue. In considering the first of these, the question becomes which enzymes are of significance. To answer this question, the illustration of tissue breakdown is shown in Figure 30. The enzymes indicated in this Figure were assayed. RNase, suggested by Berkenkamp (1973), to possibly play a role in helping pathogens establish themselves in host tissue, and acid phosphatase which is the classical marker enzyme for lysosomes, were also assayed.

The oxalate content of the exudate was also determined, because oxalate was earlier implied to act synergistically with polygalacturonase in host tissue infected with Sclerotium rolfsii (Bateman and Beer, 1965), and because it has been used in the extraction of pectic substances for some time.

The profiles for RNase, protease, β -glucosidase and acid phosphatase (Figs. 30,31) indicate that these enzymes are induced, and that they reach maximum levels (with the exception of β -glucosidase) at approximately ninety hours. These are all hydrolytic enzymes that may function in the process of autolysis, since they reach a maximum at a time when colony maturation occurs. Autolysis is usually brought about by substantial depletion of nutrients, or through inhibition of further development by the creation of an unfavourable growth environment due to the accumulation of metabolic products in concentrations inhibitory to the fungus. This is the condition which would exist at the stage of development of the colony from which these exudates are taken. Subsequent decline in enzyme concentration could be due to protease diges-

FIGURE 30

Two separate determinations of the activity of RNase in exudates from E. culmorum colonies of different ages. The substrate used was a 0.4 mg/ml solutions of yeast RNA at pH 5.5. RNase activity is expressed as μ /ml where 1 μ = the amount of enzyme required to produce a change in O.D. of 0.1 at 260 nm.

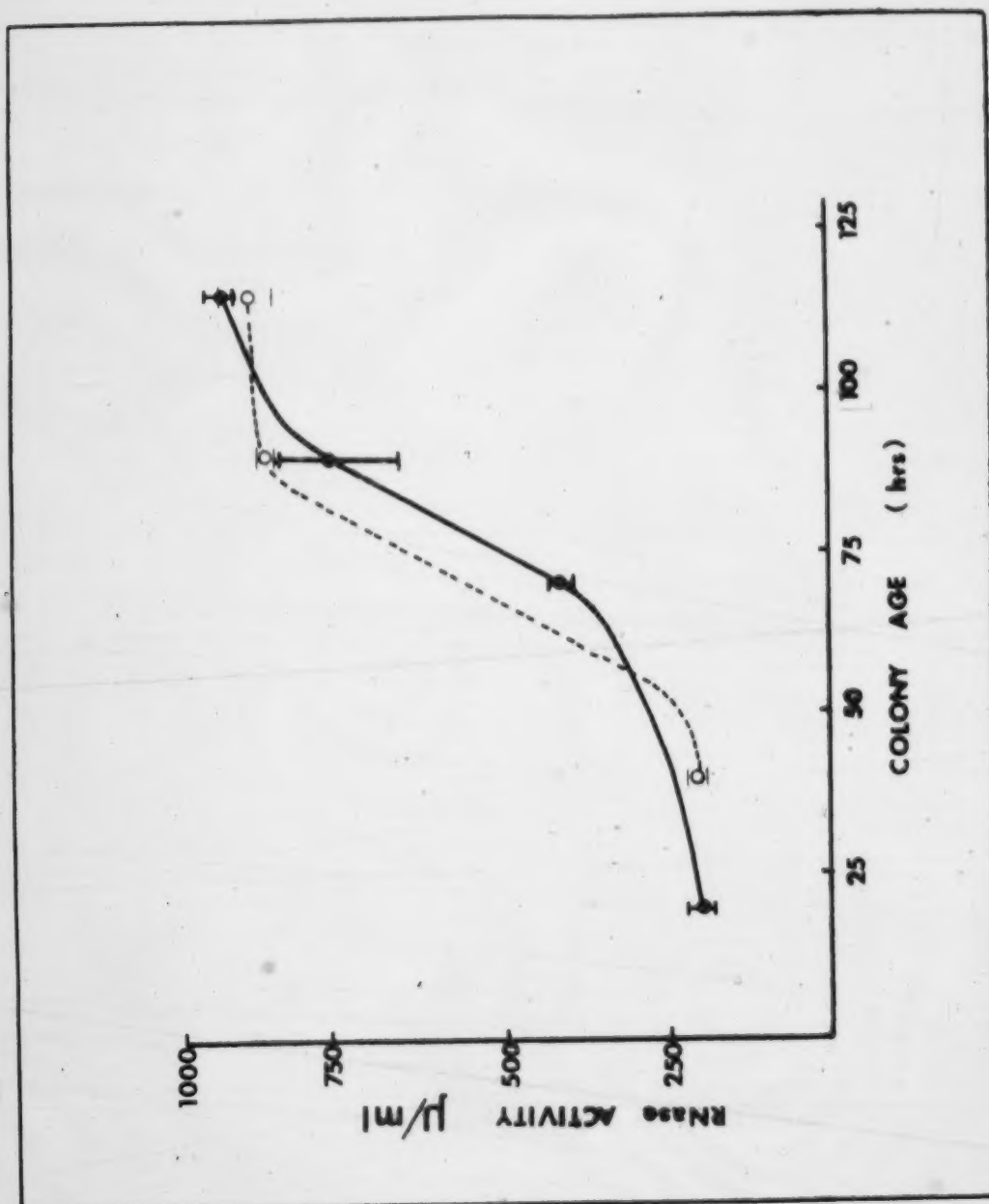


FIGURE 31

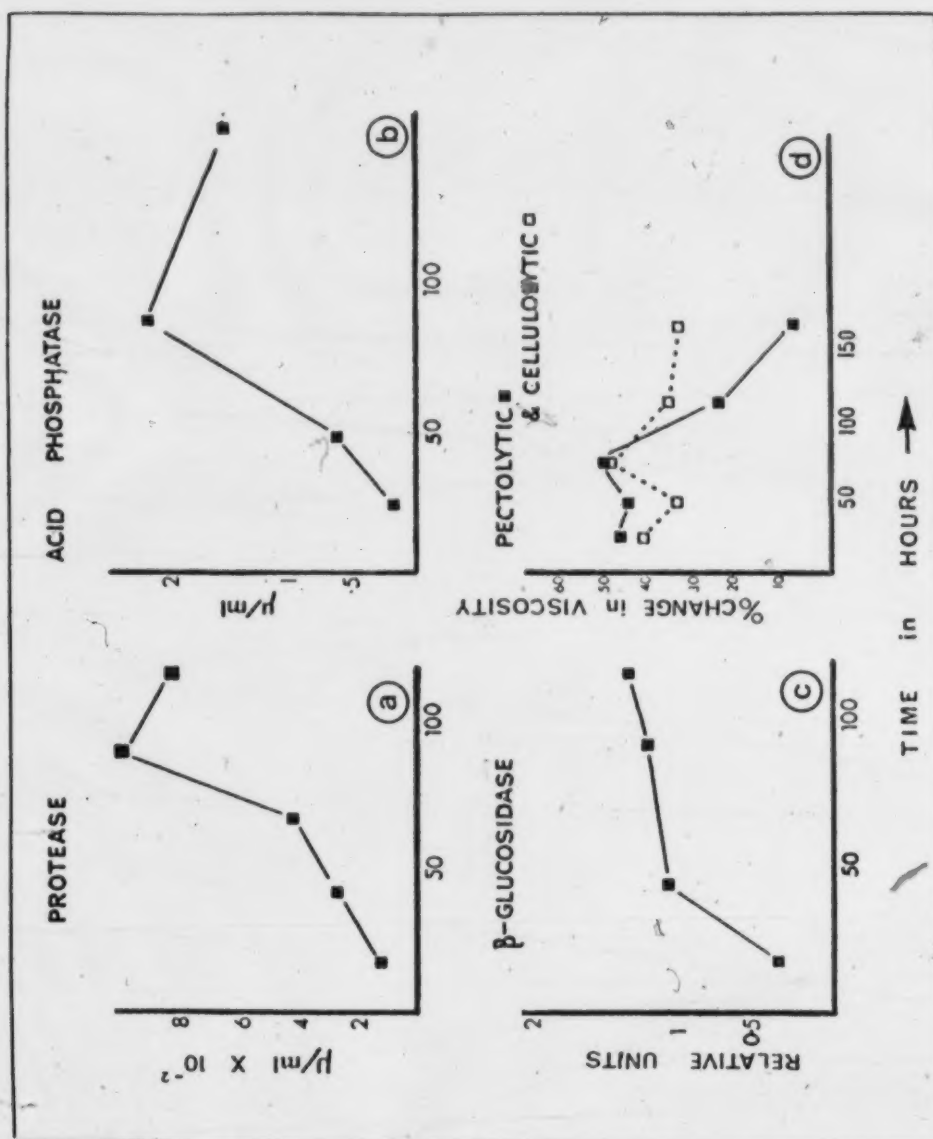
Enzyme activities in *F. culmorum* exudates from colonies of various ages.

(a) Protease activity in units /ml. Substrate was 1% casein solution in Sorenens buffer at pH 7.6. One unit of enzyme is the amount of enzyme necessary to produce an O.D. change of 0.05 at 280 nm.

(b) Acid phosphatase activity in units /ml. The substrate was p-nitrophenyl phosphate in 0.05M citrate buffer at pH 4.8. One unit of enzyme activity is equivalent to the release of $1\mu\text{M}$ of phosphate / min.

(c) Relative β -glucosidase activity determined by the colour intensity at 540 nm resulting from the release of 6-bromo-2-naphthol from 6-bromo-2-naphthol- β -D-glucopyranoside and its complexing with tetrazotized diorthoanisidine.

(d) Pectolytic (■) and cellulolytic (□) activities determined by the change in viscosity of a 0.25% solution of sodium polypectate in 0.05M sodium citrate at pH 5.5 and 0.25% carboxymethyl cellulose in 0.05 M citrate buffer pH 5.5 respectively. The activity is relative to the viscosity of the above solutions treated with equal volumes of autoclaved exudate.



tion within the exudate, although storage of exudate from Fusarium culmorum over short periods of time did not result in a decrease in protein; storage of exudate from Sclerotinia sclerotiorum did not result in loss of enzyme activity (N. Colotelo, personal communication).

β -Glucosidase did not show signs of decline at ninety hours as the previous enzyme did; but instead, indicated a continuing increase in activity within the exudate. According to Cochrane (1958), cellobiose is formed during the hydrolysis of cellulose which is substrate for β -glucosidase. Also, it is clear from Figure 3ld that cellulase activity remains relatively high for more prolonged periods; thus, the generation of substrate for the β -glucosidase could be expected.

Viscosity changes of up to 50 percent were recorded for both sodium polypectate and carboxymethylcellulose solutions treated with filtered exudate. According to Wood (1968), "many factors other than ability to produce PE* and PG** may condition pathogenesis," so that the mere presence of certain hydrolytic enzymes in vitro is not sufficient to involve them in pathogenesis. It does however, indicate a potential for tissue disintegration." No attempt was made to determine 'specific' pectin-decomposing enzymes, although it has been known for some time, that a number of such enzymes are involved in tissue disintegration.

Calcium is an essential component of cell walls, and treatment of tissues with calcium reduces the rate at which the tissues are macerated by enzymes. Glasziou (1957) hypothesized that growth regulat-

* PE = pectin methyl esterase

** PG = polygalacturonase

ing substances affect cell wall structure by altering the extent to which pectinesterase is absorbed by the cell wall. His postulate states that higher concentration of growth substances reduces the absorption of pectinesterase by the wall; thus, the pectic substances become less esterified and, in the presence of calcium, much firmer. By the same reasoning, the removal of calcium should prevent the wall from becoming firmer, and the capacity of oxalic acid to tie up the Ca^{++} which might be present, may be an important factor in the overall process of pathogenesis.

The presence of oxalic acid in the exudates was determined during colony growth (Fig. 32). According to Foster (1949), oxalate will continue to accumulate as long as the organism is alive and has available carbohydrate. Under the growth conditions used in these experiments, this would be well into the eighty hour stage, yet Figure 32 indicates a sharp drop in oxalate content of the exudate well before seventy-five hours.

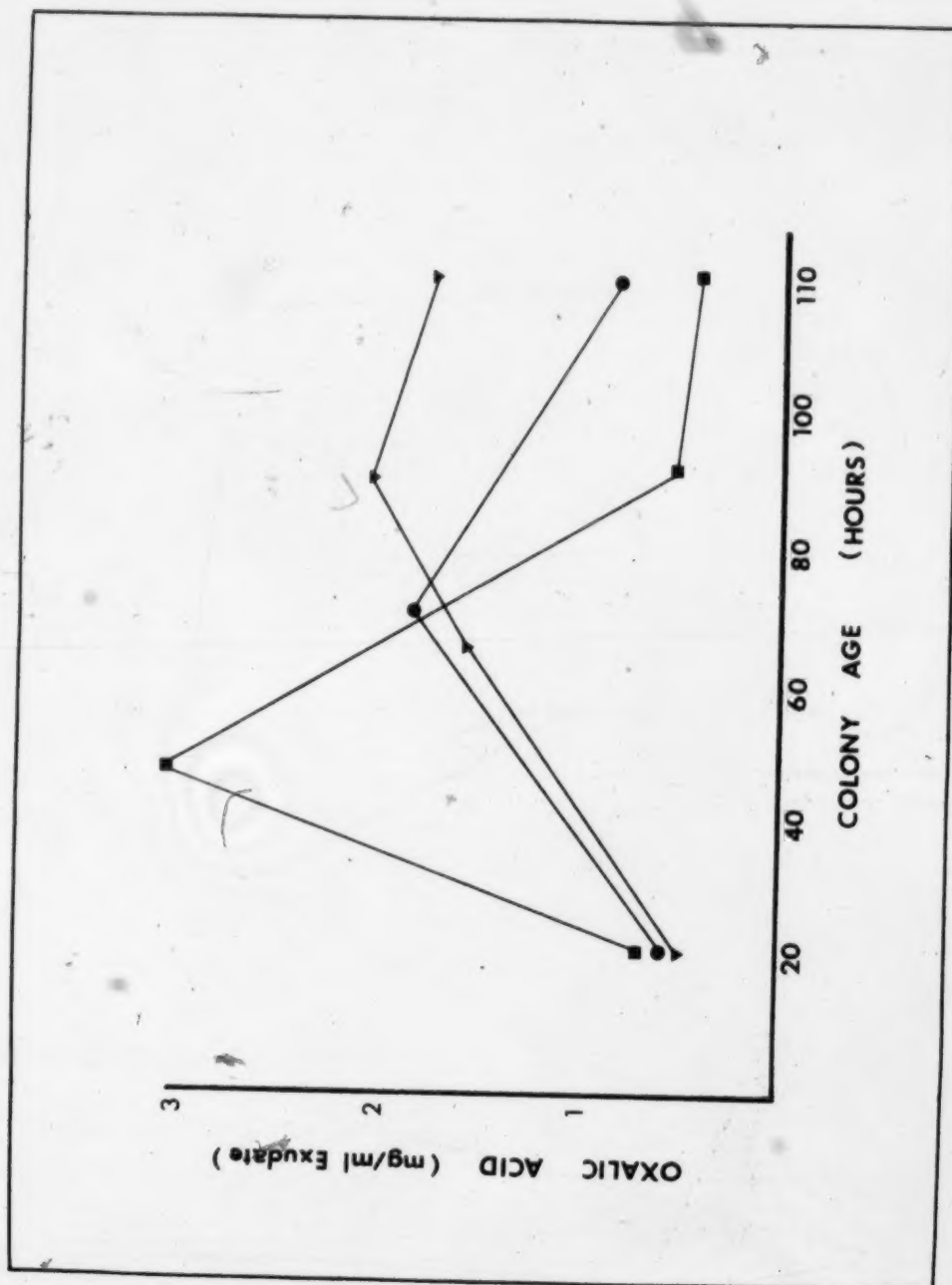
DeBary (1886) observed that the presence of calcium leads to more oxalate formation, and Robert (1911, 1912) reported that large amounts of calcium in the medium caused a 10-17 percent increase in the dry weight of Aspergillus niger, and that much of this increase was due to increased calcium oxalate deposits in the mycelium. This does offer an explanation for the disappearance of the oxalic acid, as it may be deposited in the mycelium.

The role of oxalic acid in pathogenesis is still uncertain. Oxalic acid does have the capacity to extract pectic substances from the cell wall; Bateman and Beer (1965) have suggested a synergistic action for oxalic acid and polygalacturonase. However, injections of

FIGURE 32

Oxalate content of exudates from colonies of F. culmorum of various ages. Oxalates were precipitated from solution using Ca^{++} ions, removed by filtration, redissolved and determined by titration with KMnO_4 . One ml. of 0.02N KMnO_4 is equivalent to 0.9 mg of anhydrous oxalic acid.

Each curve represents a different experimental series.



several oxalates and oxalic acid into green tomato fruit during this research, showed no visible damage to the tissue other than a very slight water-soaked appearance.

Tissue Degradation

The potential for tissue destruction by exudate has been shown in the previous section, but to demonstrate this capacity in situ, whole tissue was used. In these experiments, the response was very rapid, and the destruction of tissue extensive and non-specific. Figures 33a-f illustrate the damage which occurs to stem tissue of ninety-day-old tomato plants after seventy-two hours exposure to exudate. Although there was extensive damage to the pith region (the region into which the exudate was injected), the growing plants appeared healthy with no sign of wilt symptoms. There was no visible damage to the vascular tissue. The control, Figures 33e, f, shows negligible, visible, mechanical damage due to insertion of the needle and, Figures 33c, d show an intermediate amount of tissue damage resulting from injection of the autoclaved exudate.

The fruit of the tomato was also injected with exudate and the resultant damage recorded on film. There was variation in these results depending on whether injections were made into unripened (green) or ripened (red) tomatoes. The tissue of green tomatoes is completely disintegrated after forty-eight hours leaving a large, dry hole in the fruit, a symptom which might be generally classified as "dry rot" (black arrow, Fig. 34a). Within this cavity, there are fibres which have resisted complete breakdown (white arrows, Fig. 34a). In time-study experiments, similar damage to the tissue was observed even after

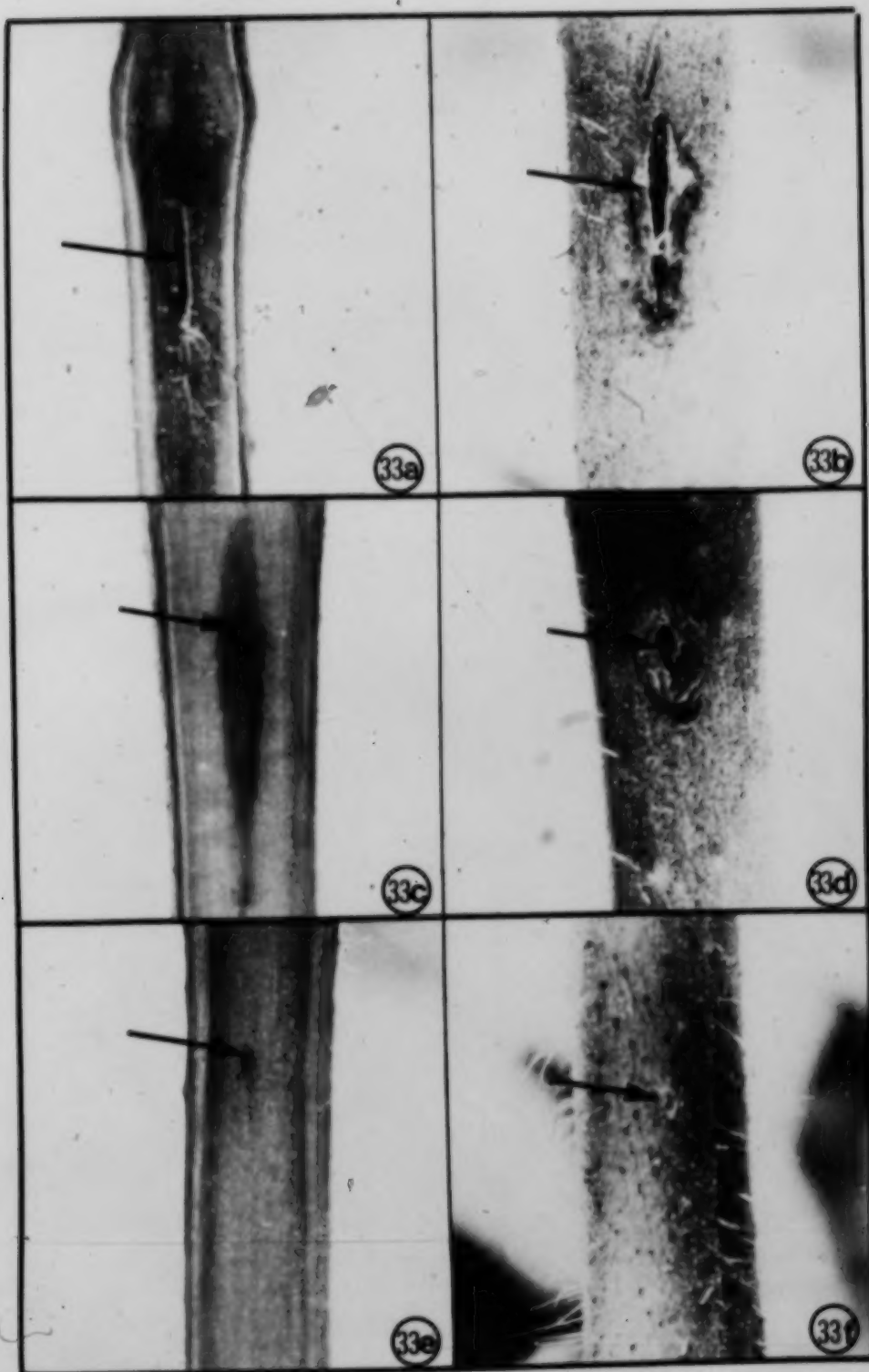
FIGURE 33

Effects of exudate, collected from mycelium of F. culmorum colonies, injected into stems of tomato seedlings:

(a, b) exudate; (c, d) autoclaved exudate; and (e, f) distilled water. Arrows indicate the point of entry of the needle.

Pictures a, c, and e are internal views of the injected areas and b, d, and f are exterior views.

In 33a (exudate), disintegration of the tissue is evidenced by a hole in the pith region and browning is evident in all the surrounding pith area. In 33c (autoclaved-exudate), browning is visible around the injection point and in the surrounding pith region. In 33e (distilled water control), browning is negligible.



only two hours. As with stem tissue, autoclaved exudate caused some water soaking, but not complete disintegration of the tissue (Fig. 34b), and damage to control was negligible (Fig. 34c).

Injection of exudate into ripe tomatoes results in a completely different response (Fig. 35). Complete disintegration, which is characteristic of the response of green tomatoes does not occur. Instead, the tissues are transformed into a mass of discontinuous cells surrounded by liquid, a condition known as maceration, and this same response is observed after injection of both green and red tomatoes with a suspension of *F. culmorum* spores. The difference in response likely reflects a difference in the cell wall structure of the two tissues involved. The fact that spore suspensions brought about soft rot symptoms in both red and green fruit may be due to:

- 1) stimulation of ripening of the tomato resulting from injection of the exudate;
- 2) the time period for germination of spores and growth of mycelium allowed for ripening of the fruit before enzyme action began.

Sugar determinations do show differences in the carbohydrate make-up of green and red tomatoes. The total reducing sugars extracted from red tomatoes were twice the content extracted from the green; TLC showed a difference in the relative amounts of glucose and fructose between extracts from green and red tomatoes. Wood (1968) states that some parasites which usually cause dry-rots, can cause lesions with soft-rot characteristics, when conditions in the host tissue are particularly suitable for growth of the parasite. One of the ways of transforming a dry rot into a soft rot is to increase the water content of the tissue. The water content of red tomatoes appears higher than the water content

FIGURE 34

Effects of exudate collected from mycelium of
F. culmorum colonies injected into green tomatoes:

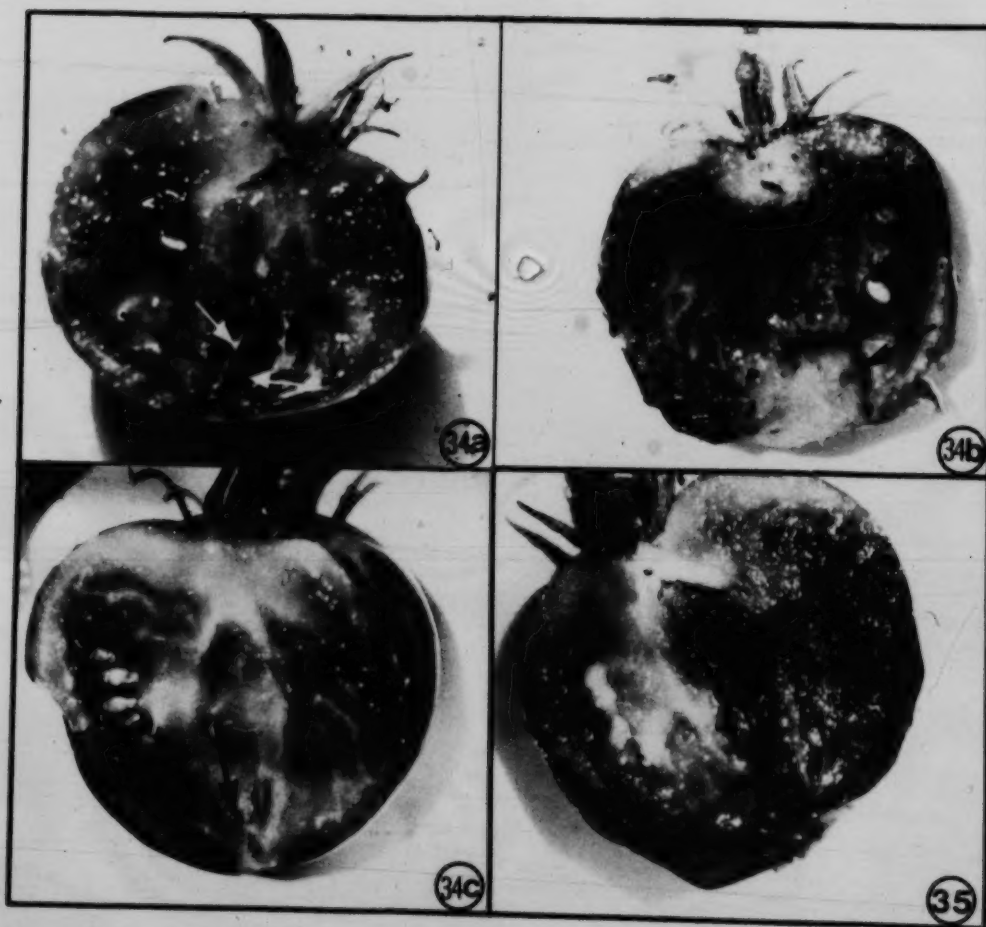
(a) Exudate. Note the hole (black arrow) and strands
of fibrous material within the hole (white arrows).

(b) Autoclaved exudate, in this case there is no
disintegration of tissue, but there is an area (light circular
zone at tip of arrow) of tissue browning.

(c) Control injection (distilled water) shows no
visible damage to the fruit.

FIGURE 35

Effects of exudate collected from mycelium of
F. culmorum colonies injected into a ripe tomato. The response
of injection of exudate into ripe tomatoes is different from
injection into green (34a). There is no disintegration in this
case but rather a maceration of tissues typical of soft rots.



of green tomatoes. Wood's postulate considers that the water content may regulate the rate of growth of the fungus; thus, "it appears that whether a soft rot or dry rot lesion develops in a plant tissue depends on how rapidly the parasite begins to secrete macerating enzymes under the prevailing conditions," (Wood, 1968). However, the enzyme complex used here for injection into red and green tomatoes was identical; therefore, it is concluded that in this case, the difference in response reflects a difference in the biochemical make-up of the host tissues.

Primary wall is prevalent in younger tissue and the arrangement of the cellulose in primary wall is relatively unbound, so reversal of wall structure is generally possible at this stage. By comparing these conditions to the secondary wall, which is composed of closely packed microfibrils and has a permanent rigidity, it is easy to see the relative ease with which the younger tissue is completely disintegrated. Enzyme attack of the secondary wall would tend to result in destruction of the middle lamella causing intercellular dissociation or maceration, but the cells would still maintain an intracellular cohesion. This does occur with older tissue.

Sunflower tissue responded to exudate injections in a similar manner. Figure 36a, b shows a complete disintegration of tissue in the area of the injection; autoclaved exudate results in browning (Fig. 36c) and water soaking but no disintegration; distilled water has little visible effect (Fig. 36d).

During these disintegration experiments, it was noticed that exudates collected in the earlier stages (up to forty hours) produced a white flocculent precipitate upon autoclaving, and the precipitate was included in the autoclaved injections as a suspension. The pre-

FIGURE 36

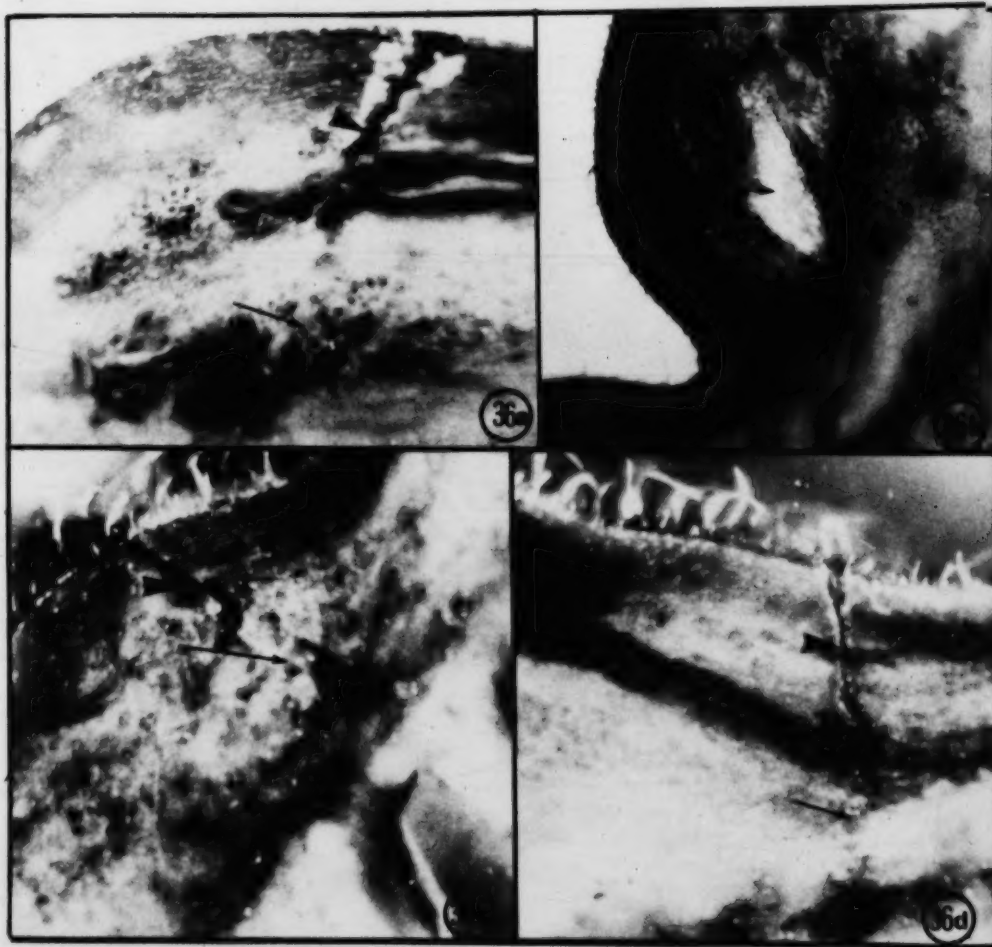
Effects of exudate, collected from mycelium of F. culmorum colonies, injected into sunflower heads. In each case the arrow head indicates the needle-entry scar:

(a) Exudate injection causes complete disintegration of the tissue (note the spongy texture of this tissue).

(b) A thin section through the area indicated by the arrow in (a) shows the extent of the hole formed.

(c) Autoclaved exudate produces an area of tissue browning (small arrow).

(d) Control (distilled water) causes negligible damage in the region of the injection (small arrow) and the tissue layers are relatively ordered compared to those in (c).



precipitate visually resembled protein, but analysis of the autoclaved material showed that the filtrate had the same protein level as non-autoclaved exudate. Analysis did show a slight quantitative loss in reducing sugars after autoclaving. Separate injections of the supernatant and the washed resuspended precipitate, demonstrated that the active component in the autoclaved sample is contained in the precipitate.

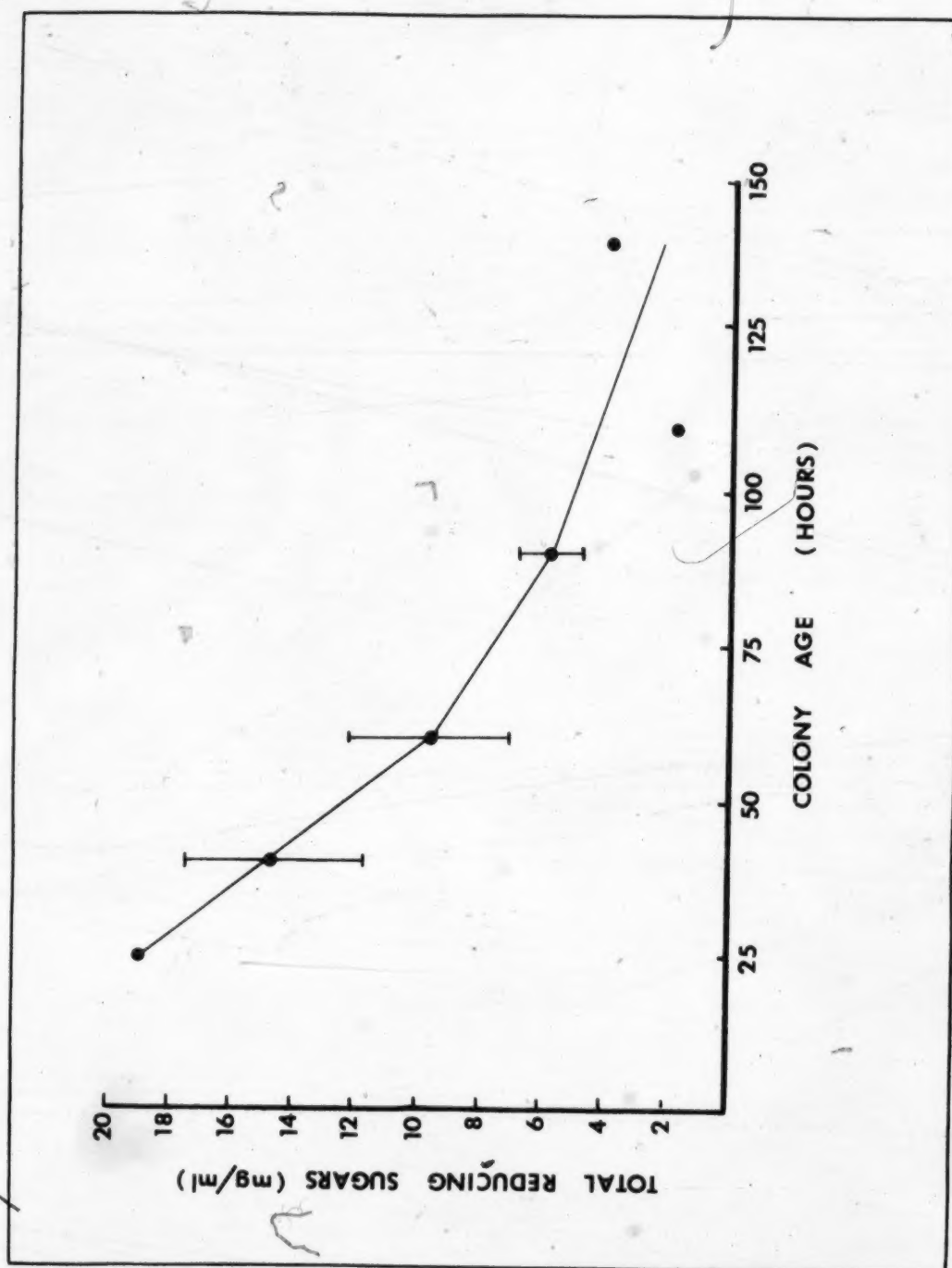
When this precipitate was hydrolysed overnight in 2 N HCl at 100°, then tested for reducing sugar and amino acid content, both were found to be present in significant amounts. It is felt that perhaps this precipitate is a glycoprotein, since glycoprotein has been detected in mycelial extracts of ground, fresh mycelium of Aspergillus fischeri (Gorcica et al., 1934), in association with yeast cell walls (Kom and Northcote, 1960) and in association with an enzyme system of Aspergillus niger (Zaborsky and Ogletree, 1974). A protein-lipo-polysaccharide complex has been implicated with fungal pathogenesis in relation to Verticillium wilt of cotton (Keen, Long and Erwine, 1972; Keen and Long, 1972; Partridge and Zaki, 1972).

The total reducing sugars in exudates collected from colonies of various ages was determined (Fig. 37). The concentration, initially relatively high, dropped off rapidly as the colony overgrew the petri plate. The presence of sugars in hyphal exudates can be expected, based on the theory that these exudates are related to the lysosomal system and are found in the tip region, which is a region of very active wall synthesis.

A qualitative analysis of the sugars present was carried out by thin layer chromatography and is reported in the section on "Specific Effects of Exudate on Potato Tissue".

FIGURE 37

Total reducing sugars in exudates collected from F. culmorum colonies of various ages as determined by the Somogyi's colourimetric method. Total reducing sugars were determined as glucose equivalents from a glucose standard curve. (Standard deviation bars are shown).



Specific Effects of Exudate on Potato Tissue

To relate previous findings to the disintegration of potato tissue, exudate was reacted directly with potato discs cut from the storage tissue of raw tubers. The losses in both wet and dry weight were evaluated. A dry weight loss was recorded (Fig. 38), indicating removal of solid material from potato cells as a result of the action of both autoclaved and non-autoclaved exudate. The effect of buffer alone is negligible. However, wet weight determinations (Fig. 39) show a different response, in that only non-autoclaved exudate show a significant weight loss. For a possible explanation, it is considered that the dry weight of potato tissue is approximately 20 percent of the total weight in the tuber; a 30 percent loss in dry weight constitutes approximately a 6-7 percent loss of total weight. This is in the range of the small amount of wet weight loss shown for autoclaved exudate (Fig. 39). Non-autoclaved exudate-treated tissue shows a 30 percent loss in wet weight and for a similar reason, only 1/3 of this is accounted for as loss in dry weight. The conclusion is that a large portion of the wet weight change, shown by non-autoclaved-exudate-treated discs, is due to an efflux of liquid from the cells, which may indicate an alteration in cell membrane permeability. An alteration of cell membrane permeability, in relation to pathogenesis, was discussed in this thesis in the section "Tissue Degradation" and may be a significant factor in the death of cells.

Observation of the discs after treatment showed an obvious visible difference between treatment with exudate and straight buffer. The exudate treated discs were thin, soapy to the touch and lacked rigidity (the latter property may be due to the efflux of liquid mentioned). The

FIGURE 38

Effect of 0.1 ml of exudate from 60 hour colonies of *E. culmorum* on the dry weight of 20-disc potato samples incubated with 0.5 ml of citrate buffer, pH 5.5 and temperature 20°. The dry weight of a 40 disc sample taken at 0 time = 100%. The three graphs represent three individual experiments carried out under equivalent experimental conditions. Discs were dried overnight at 95°.

- = exudate
- = buffer control
- △ = autoclaved exudate

Dashed lines represent extrapolation to zero time.

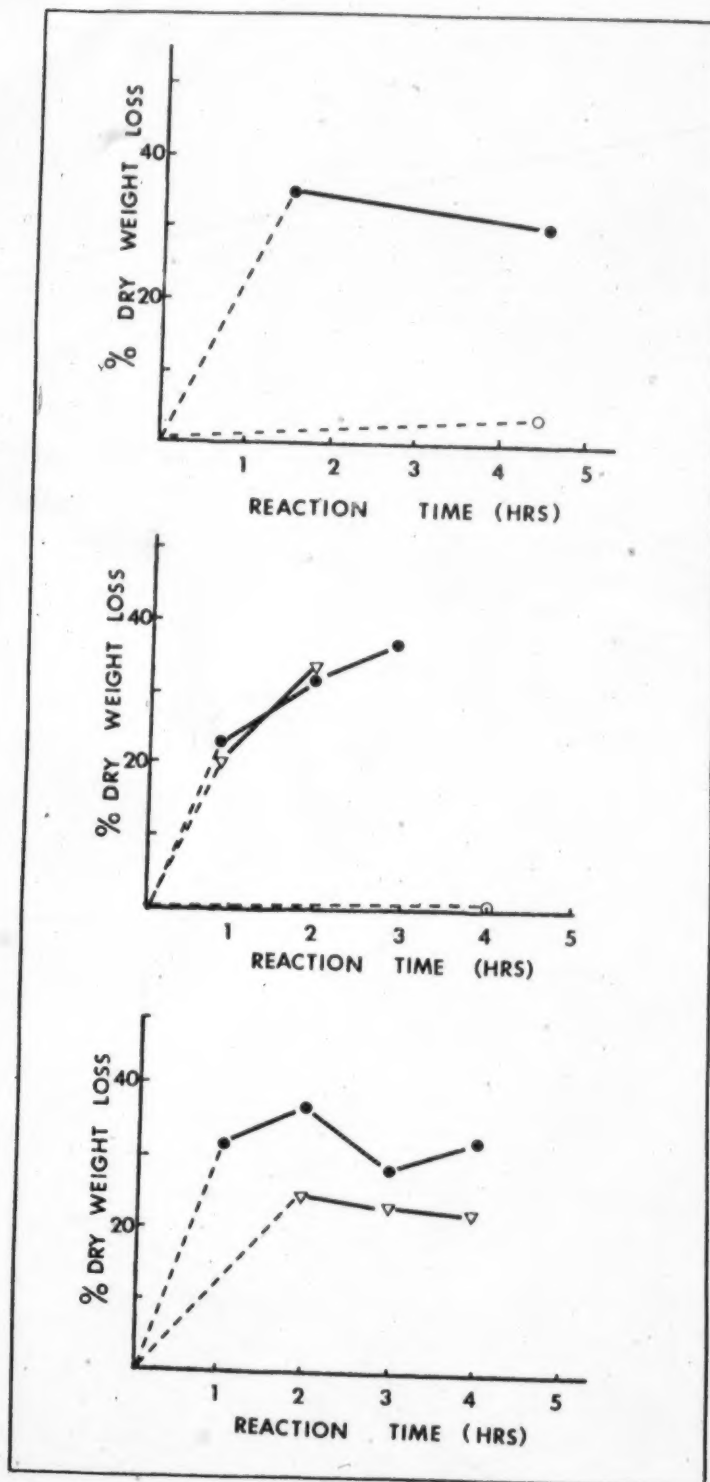


FIGURE 39

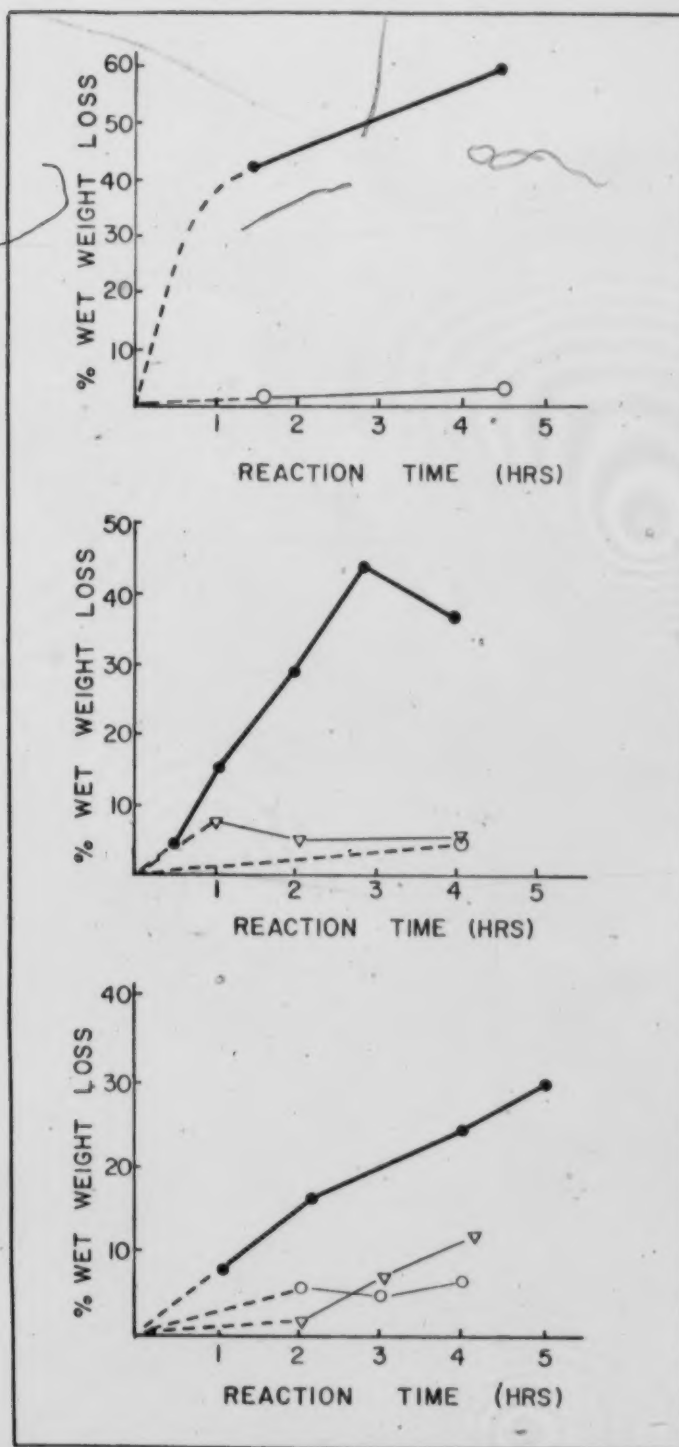
Effect of 0.1ml of exudate from 60-hour colonies of F. culmorum on the wet weight of 20-disc potato samples incubated with 0.5 ml. of citrate buffer, pH 5-5 and temperature 20°. The set weight of a 40-disc sample, taken at 0 time, = 100%. The three graphs represent three individual experiments carried out under equivalent experimental conditions. The wet weight was the weight of the discs after they were removed from solution and excess water blotted off between two paper towels.

● = Exudate

○ = Buffer

▽ = Autoclaved exudate

Dashed lines represent extrapolation to zero time.



discs treated with autoclaved exudate on the other hand, remained rigid and "wafer-like", i.e. maintained their turgor as did the controls.

A more detailed look at the alteration of potato cell wall as a result of exudate treatment was possible using the scanning electron microscope. Figure 40a-d shows a potato cell wall which has been exposed to exudate for various time intervals. This series of electron micrographs indicate that under the experimental conditions used:

1) the cell wall is altered by exudate treatment; 2) the action of the exudate is rapid and damage to the secondary wall matrix is extensive; and 3) the fibrils within the wall are resistant to the action of the enzymes involved.

The unaltered cell wall (Fig. 40a) is relatively smooth, and although ribbed, appears to be homogeneous. In comparison, many electron micrographs of plant cell walls show the cell wall surface to be fibrous. This is true for the primary wall of parenchyma cells from onion bulbs (Robards, 1970), the secondary wall of mature vesicles in carrot cell walls (Steward, 1968), the primary walls of parenchyma tissue from wheat (Preston, 1964), and the parenchyma cells of Avena coleoptile (both inner and outer surfaces) (Wardrop, 1955, 1956). Generally, in isolation of cell walls, the cells are macerated and treated with 1N NaOH and 1N HCl; the hydrolytic action would remove some of the hemicellulose and pectic material of the matrix and expose the underlying fibrils. The only treatment used on the cells shown in Figure 40a,b, other than the usual fixation procedure, was 1/2 hour in 0.05M citrate buffer at pH 5.5. The fibrils in this case, are not visible until after the cell wall matrix has been removed by enzyme digestion.

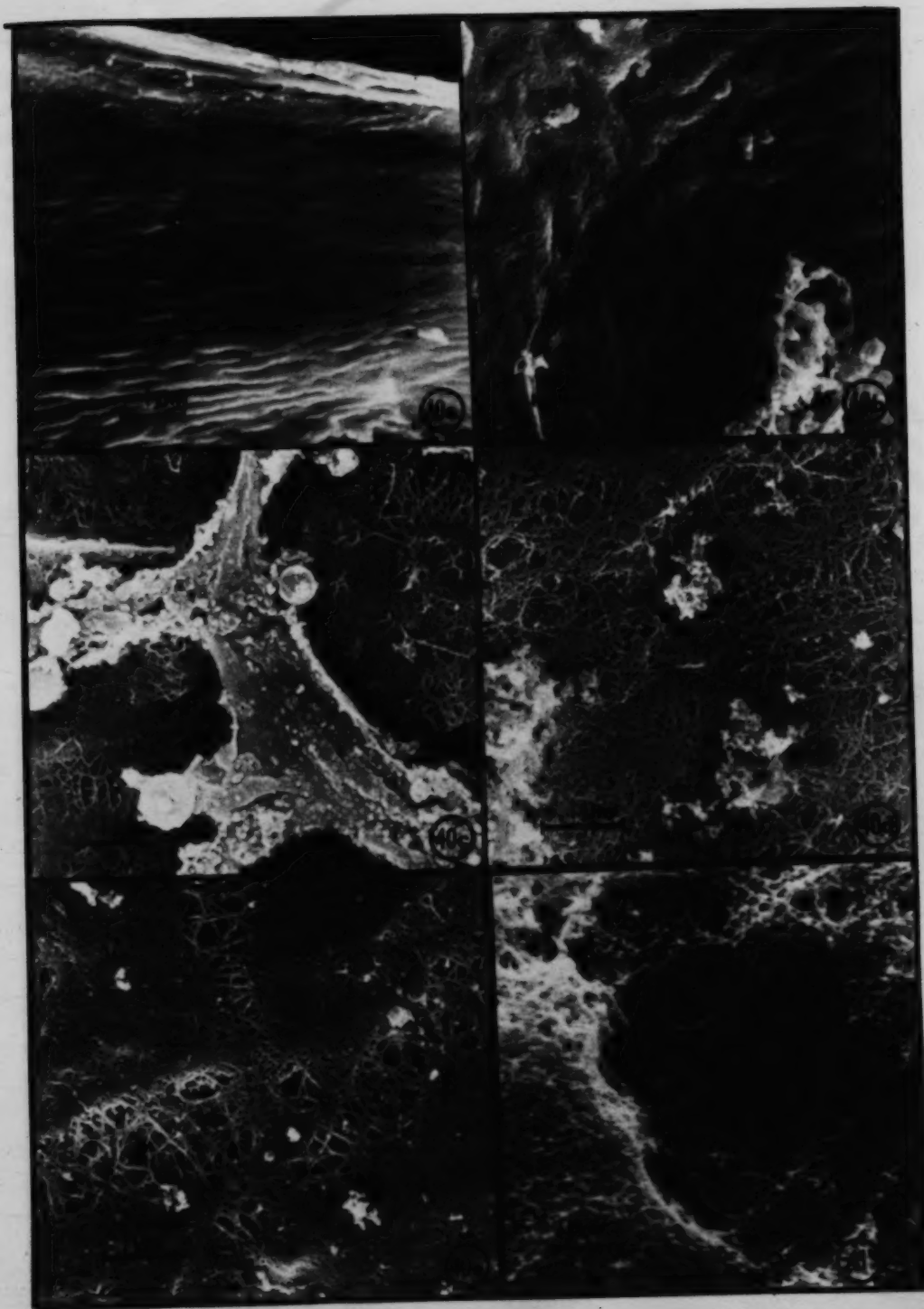
FIGURE 40

Scanning electron micrograph of potato tissue treated with exudate from a 48-hour colony of F. culmorum:

- (a) Cell wall of potato tissue incubated for 1 hour. 20 Discs were incubated in 0.6 ml of 0.05 M citrate buffer, pH 5.5.
- (b) Buffer treatment (as in "a") for 24 hours.
- (c) Cell wall of potato tissue incubated 1 hour with exudate. 20 Discs were incubated in 0.1 ml exudate + 0.5 ml of 0.05M citrate buffer, pH 5.5.
- (d) Potato cell wall which has been exudate treated for 5 hours (same conditions as in "c").
- (e) Potato cell wall which has been exudate treated for 24 hours (same conditions as in "c").

FIGURE 41

Scanning electron micrograph of a section of potato tuber tissue with advanced dry rot symptoms.



Potato discs treated with autoclaved exudate do not show the dissolution of matrix material which is seen with non-autoclaved exudate.

From Figure 40c, it appears that approximately 25 percent of the matrix is removed from the surface layer in only one hour. The damage occurring after twenty-four hours does not appear to be much greater in relation to the surface area affected, but it may penetrate considerably deeper. The micrograph of tissue from potato infected with a Fusarium sp. and showing advanced dry rot symptoms (Fig. 41) does show similar random fibril arrangement to exudate-treated tissue (Fig. 42), but also indicates deeper and more complete removal of the matrix material.

Exudate-treated discs are much less rigid than those treated with buffer. Figure 42 illustrates physical distortion of cells which occurs after treatment of the discs with exudate. The nature of this distortion suggests that the matrix is necessary to maintain the rigidity of the cell wall.

A model for the structure of cellulose fibrils within the cell wall has been proposed by Robards (1970) (Fig. 43). The author states that in many cases, each microfibril may have only a single crystallite (micelle) and in that case, the diameter of a macrofibril would be in the range of 600-700⁰Å. The 'macrofibril' in Figure 43 is approximately 700⁰Å. Recognition of fibrils of polygalacturonic acid on the surface of cultured cells of several species (Leppard et al., 1971) indicates that cell wall fibrils can no longer be assumed to be only cellulose in nature. However, a comparison of the one hour and twenty-four hour exudate-treated discs (Figs. 40c,e) shows no visible difference in the

FIGURE 42

Scanning electron micrographs of potato discs treated with exudate from F. culmorum.

(a) Surface view of a potato disc which has been suspended for one hour in 0.05M citrate buffer at pH 5.5.

(b) Surface view of a potato disc which has been suspended for one hour in exudate solution (0.1 ml exudate + 0.5 ml of 0.05M citrate buffer, pH 5.5).

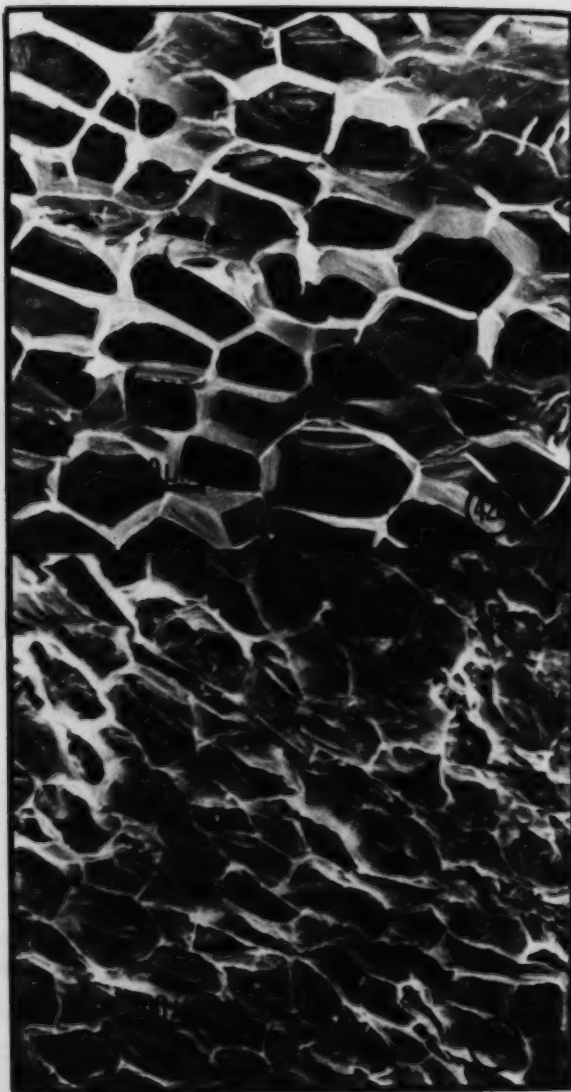
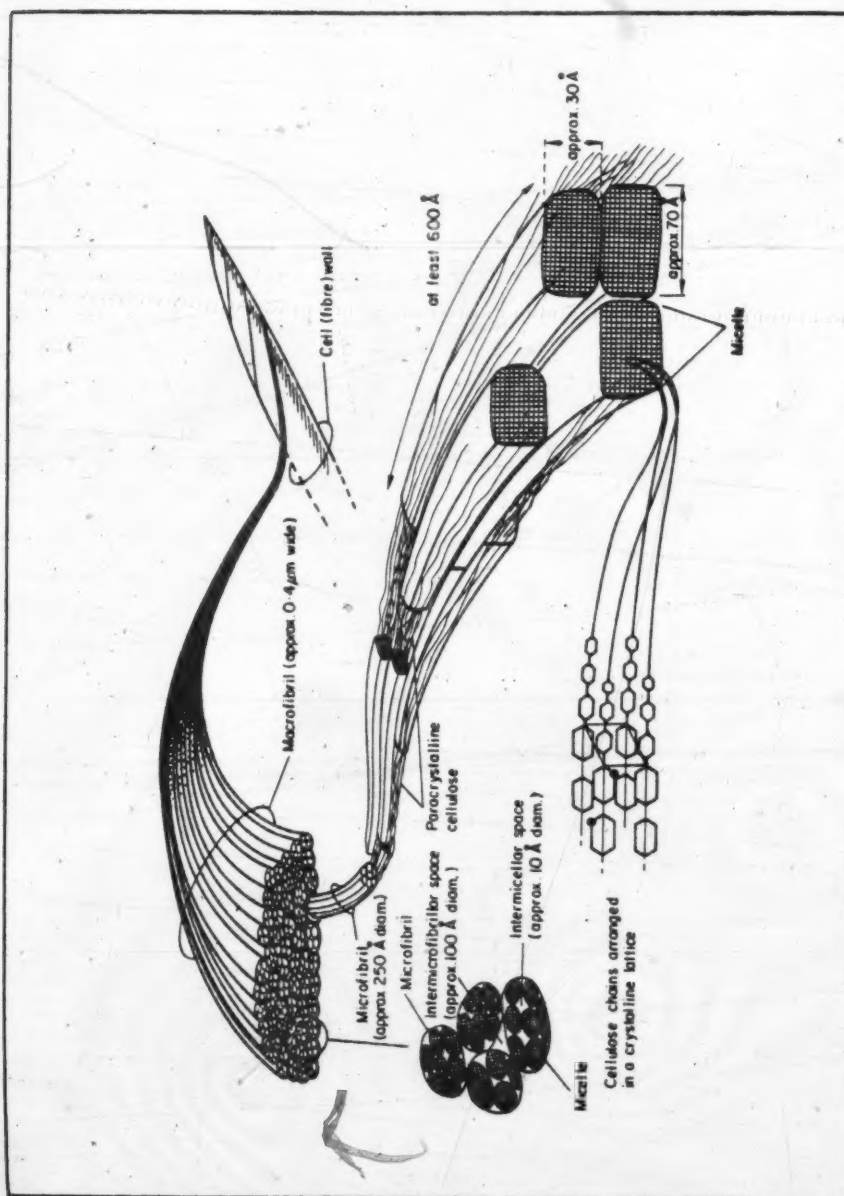


FIGURE 43

Schematic diagram showing the arrangement of cellulose in cell walls. This diagram shows how a complete cell wall is built up from the individual chains of cellulose. Each microfibril is shown to be comprised of about 6 micelles, but in many cases, each microfibril may have only a single crystallite (micelle).

(From Robards, 1970)



structure of the fibres. This indicates that they are not attacked by the enzyme system of the exudate. Tissue from a potato specimen suffering from natural dry rot also shows little obvious breakdown of the exposed fibrils (Fig. 41). This may indicate that these fibrils are cellulose.

The cell wall materials released during exudate digestion were investigated qualitatively using thin-layer chromatography. A "fingerprint" of the sugars in the exudate was first established to determine what sugars would be introduced into the system. The results of these TLC runs are shown in Figure 45. The sugar pattern indicates that the following are present: glucose, sucrose, trehalose, fructose and glucosamine. The shape and position of the "glucose spot" suggests that it may be a combination of sugars. Comparison of this "glucose spot" with the standards (Fig. 46, 47) suggests mannose is present. Marchant (1966) found galactose, glucose, mannose, arabinose and xylose in the conidial cell walls of *F. culmorum* as well as the amino sugar glucosamine.

Quantitative data for total reducing sugars show a decrease in sugar content as the age of the colony from which the exudate was harvested increases, but the TLC profiles (Figs. 44, 45) show the same "fingerprint" for 40, 69 and 90 hour collections of the exudate.

A one dimensional thin-layer chromatogram (Fig. 48) shows an increase in the amount of material released from the potato discs with time. There are a number of spots that appear, particularly below the fructose spot, after two hours of incubation in the presence of exudate (Fig. 48). Two dimensional chromatograms of similar material show the presence of a spot corresponding to the galacturonic acid

FIGURE 44

A one-dimensional thin-layer chromatogram run on exudate from 40-hour (right hand side of plate) and 65 hour (left hand side of plate) colonies of F. culmorum. In the centre is a run of a 0.3 mg/ml standard mixture of glucose, sucrose and fructose. The chromatogram was developed in n-propanol: ethyl acetate: water, 7:1:2 for 5½ hours at 25° and stained with a 5:5:1 mixture of 4% ethanolaniline: 4% ethanolic diphenylamine: 85% phosphoric acid. The origins (indicated by arrows) were spotted with 10 µl aliquots.

FIGURE 45

Sugar "fingerprints" of exudates from colonies of F. culmorum of various ages run on two-dimensional thin layer chromatograms. The first dimension was run and developed under the same conditions outlined in Figure 44. The second dimension was developed in acetone : formic acid : ethanol (95%), 3:1:1 for 1½ hours. The origins in the lower right hand corner (indicated by a O) were spotted with 10 µl. aliquots of a $\frac{1}{10}$ dil $\frac{m}{m}$ of exudate

- (a) Exudate collected from a 40-hour colony.
- (b) Exudate collected from a 69-hour colony.
- (c) Exudate collected from a 90-hour colony.

The spots are numbered according to position and colour (see Figure 47).

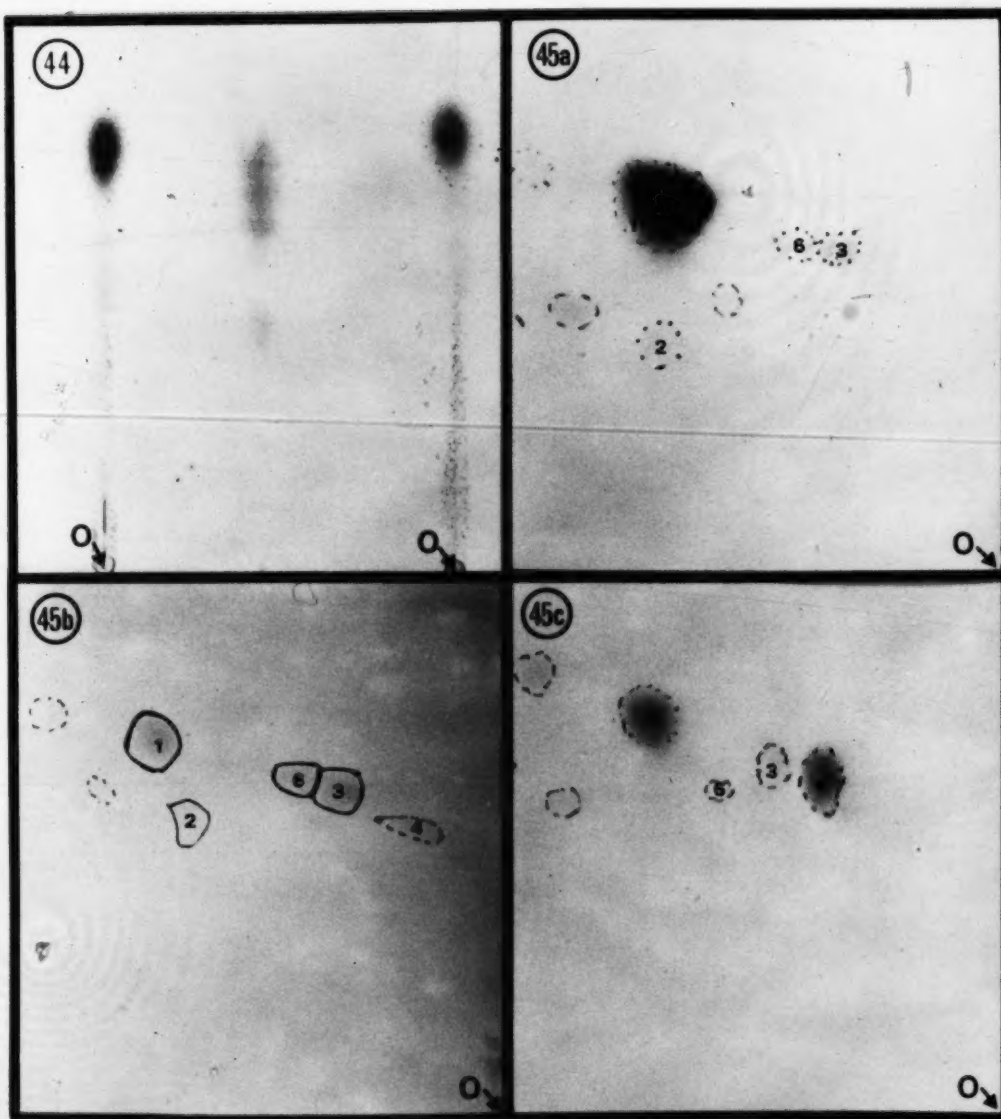


FIGURE 46

Two-dimensional thin layer chromatograms of the sugar standards. Standards were applied in the lower right hand corner of each plate at the point indicated by an O. Experimental conditions were the same as in Figure 45.

(a) Mixture of glucose (1); fructose (2); sucrose (3), and trehalose (4).

(b) Mannose (7). B = blue

(c) N-acetylglucosamine (6). Br = Brown

(d) Galacturonic acid (5). LB = Light blue.

Standard concentrations were 1mg/ml and they were applied undiluted.

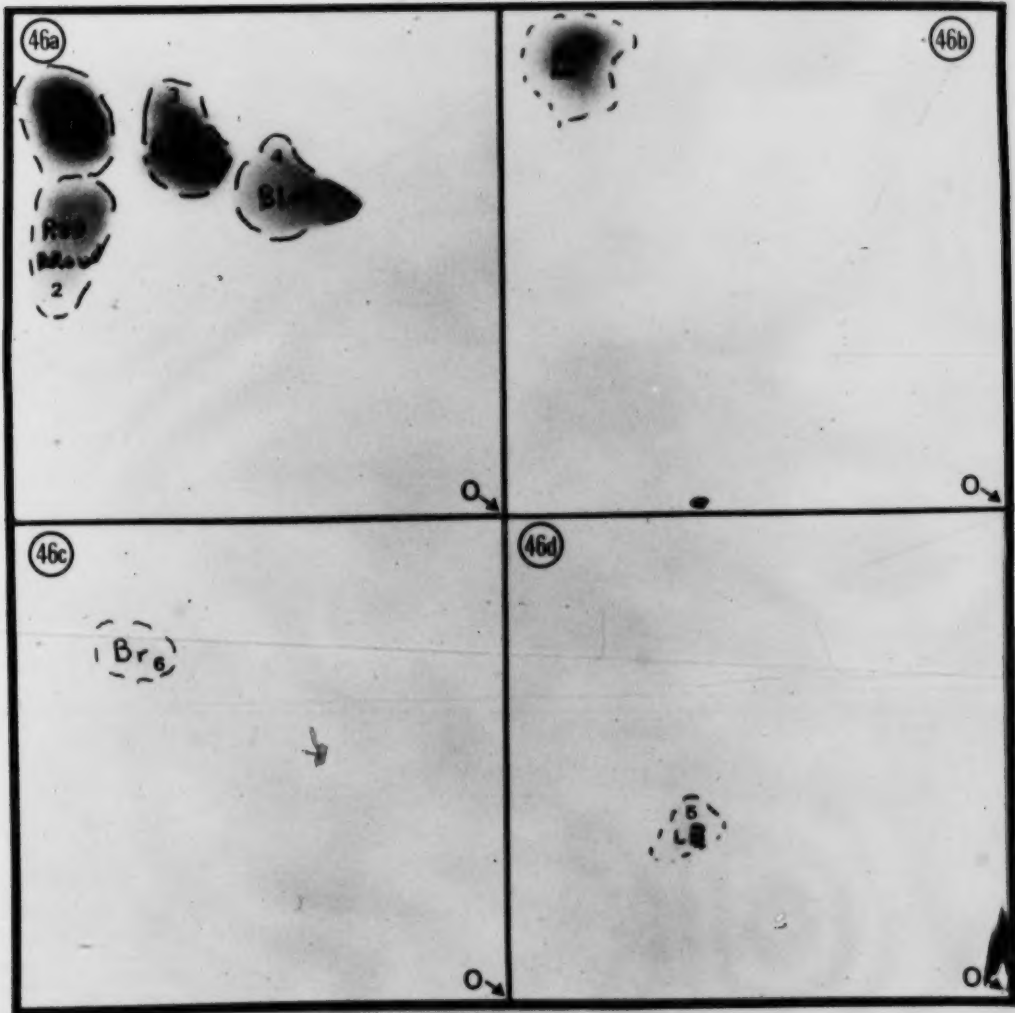


FIGURE 47

Composite diagram showing positions of standard
sugars on the two-dimensional thin layer chromatograms shown in
Figure 46.

LEGEND

- | | |
|----------------------------------|--------------|
| 1. glucose | dark blue |
| 2. fructose | red - brown |
| 3. sucrose | green - grey |
| 4. trehalose | blue |
| 5. α -D-galacturonic acid | light - blue |
| 6. N-acetylglucosamine | brown |
| 7. mannose | blue |



FIGURE 48

A one-dimensional thin layer chromatogram showing sugars found in the supernatant solution from potato discs treated with exudate from 92-hour *F. culmorum* colonies. The runs, from left to right, are:

Blank (potato discs + buffer) after 1/2 hours.

Blank (potato discs + buffer) after 24 hours.

33-1, 1/2 (potato discs + exudate + buffer) after 1/2 hour.

33-1, 2 3/4 (potato discs + exudate + buffer) after 2 3/4 hours.

33-1, 24 (potato discs + exudate + buffer) after 24 hours.

Experimental conditions for thin layer chromatography are the same as those in figure 44. Ten μ l aliquots were spotted at the origin (see arrows).

FIGURE 49

Two-dimensional thin layer chromatogram showing the sugars found in the supernatant solution from potato discs treated with exudate from 42-hour *F. culmorum* colonies. Experimental conditions for thin layer chromatography are the same as those in Figure 45. Ten μ l aliquots were spotted at the origin (small arrow). The large arrow indicates the position of a faint galacturonic acid spot. The spots are numbered according to position and colour (see Figure 47).

standard (Fig. 49). This is a clear indication of the breakdown of pectic material and its release from the cell wall, and confirms the interpretations based upon the scanning electron micrographs.

CHAPTER IV

GENERAL DISCUSSION

The role of exudates in pathogenesis was the major concern of this research. First however, it was necessary to establish some basic concepts related to the process of exudation. The technique developed for collection of the exudate allows separation of the liquid from the mycelial mass and independent evaluation of it. The approach is new, and introduces a system in which the biochemical parameters can be separated from the physiological parameters, at least as far as the pathogen is concerned. However, it is necessary to keep in mind that such a system is an artificial one and caution must be used when interpreting results.

Experimentally, the system used here interacts fungal exudate with host tissue. By-passed are the penetration and infectivity stages, both of which are highly significant in the process of pathogenesis. However, the inter- and intracellular interaction between fungal pathogen and host is initially one of exo-metabolite interaction (Barnett, 1974; Curtis and Barnett, 1974) and the influence of fungal pathogen on host tissue is well in advance of the fungal pathogen. Thus, injection of exudate does parallel some segments of the process of pathogenesis, even though it is only one isolated part of the system.

The general equation of pathogenesis (Fig. 29) indicates that pathogen-secreted polysaccharide-degrading enzymes play a fundamental

role in pathogenesis. Increased knowledge of the structures of the substrates of these enzymes, that is, the polysaccharides of plant cell walls, has advanced the concepts concerning their role in pathogenesis. Albersheim et al. (1975) has refuted the importance of cell wall composition of host in the induction of specific polysaccharide degrading enzymes by pathogens. He feels that similarities between cell walls of different plant species, and the fact that the walls of all plants are composed of the same ten or so monosaccharides, is evidence that the cell wall structure is not a significant influence in pathogenesis. He also suggests that polygalacturonase involvement in the degradation of cell walls is a general phenomenon independent of the particular host variety.

A theme of this thesis has been that the process of exudation and thus, the contents of the exudate, are related to the normal function of the fungal colony and therefore independent of the particular host. It follows that the presence of cell wall degrading enzymes allows the pathogen an advantage in a susceptible host so that production of certain cell wall degrading enzymes is a prerequisite for parasitizing a particular host.

Albersheim et al. (1975), in their review, discount the importance of possible minor differences in cell wall glycosyl linkages, but it seems to this author that this is a very weak assumption. Induction of specific enzymes by specific substrates is well documented (Christensen, 1951; Cooper and Wood, 1973; Singh and Wood, 1956) as well as the role of simple sugars in the control of enzymes during pathogenesis (Cooper and Wood, 1973; Horton and Keen, 1966).

The influence of cell wall mono-, di-, tri-, and polysaccharides may be a quantitative phenomenon as well as a qualitative one so that the success of a particular pathogen may depend on the release of sufficient quantities of a particular saccharide. It appeared from the results of injection of exudate into green and red tomatoes that response is probably due to differences in cell wall composition.

A systematic approach to a problem, such as the influence of cell wall on pathogenesis, may be possible. A defined natural enzyme system (this could be done for the exudate collected from a pathogen) would allow reconstruction of the enzyme system, one enzyme at a time. Scanning electron micrographs of the tissue would show any major changes in cell wall structure due to the action of the enzyme(s) employed, and this could be correlated with analysis of cell wall breakdown products as was done in this thesis for exudate treatment of cell wall.

Some gas-liquid chromatography was done for qualitative and quantitative analysis of released sugars with promising results. Further details of this have not been given because of the preliminary nature of the work. The use of SE30 packing in a 4 foot glass column on a Barber-Coleman Model gas chromatograph gave excellent separation of a number of silylated mono- and disaccharides and tentative confirmation of the TLC results on exudate sugars.

It is possible that slight variations in cell wall polysaccharides may be genetically controlled. Major genetic mutations undoubtedly result in plant death, but minor mutations can result in varietal differences. These differences, if they represent differences in glycosyl linkages or polysaccharide sequences, may represent a basis

for resistance to particular pathogens and with the proper biochemical data, the plant breeder could use this information for selection of resistance.

Other resistance mechanisms involve specific enzyme inhibitors (Albersheim et al., 1971) and elicitor-phytoalexin systems (Keen et al., 1975), and information on cell wall structure and interaction with pathogen exudate will undoubtedly aid in interpretation of these related areas.

This release of liquid from fungi is involved in systems other than the pathogen-plant system. For example, Aspergillus flavus is a human pathogen, and it also excretes liquid droplets similar to those seen on Fusarium culmorum (N. Colotelo, personal communication). The role of these droplets in the pathogenicity of this fungus should be considered. Penicillium spp. exude liquid which collects on the colony surface and which is, in some cases, characteristic of the particular species. The species of Penicillium which produce penicillin, also, produce yellow droplets on the surface of the colony. When assayed, these drops show penicillin amounts closely approximating the amounts present in the broth (K. B. Raper, personal communication).

Ergot toxicity has been known to man for much of recorded history, yet mycotoxicity remained a neglected area until the 1960's (Kadis, Ciegler and Ajl, 1972), and the role as causative agent of disease of many of the mycotoxins remains to be determined. Many of the fungal toxins associated with animal and human foods show up as a result of contamination by plant pathogens. Two coumarin derivatives that possess strong phytotoxic properties have been isolated from celery infected with Sclerotinia sclerotiorum (Scheel et al., 1963).

Smalley et al. (see Mirocha and Pathre, 1972) found that Fusarium tricinctum was one of the fungi most frequently isolated from corn associated with toxicity in domestic animals in Wisconsin and Ueno et al. (1973) has listed thirteen Fusarium species showing lethal toxicity in mice.

It is possible in the above cases that the toxic component is exuded by a system similar to the one described here (F. culmorum was one of the species included by Ueno (1973)).

Classically, evaluation of the toxic component or destructive capacity of a pathogen has been carried out using culture filtrates. Brown (1915), working with Botrytis sp. initiated the use of culture filtrates and since that time the technique has been routinely used (Bateman and Beer, 1965; Horton and Keen, 1966; Cooper and Wood, 1973). The exudate system utilizes the selective permeability of the fungal membrane and is isolated from the staling products or products resulting from chemical interactions within the culture filtrate.

In plant pathogenesis, as the pathogen advances into the host, active excretion of exudate is always occurring, and the exometabolite environment is therefore always 'fresh'.

Summary

The presence of liquid droplets on aerial, filamentous mycelia is a general feature of fungal colonies growing in nature or on artificial culture media, yet only a few references to the phenomenon are recorded (Fenner, 1932; Raper and Thom, 1968; Thom, 1930), and even then, only casual mention is made. A considerable amount has been reported in relation to exudation from fungal reproductive structures (Buller, 1958; Knoll, 1912; Remsberg, 1940) and more recently, work

has been done on the biochemistry of these droplets (Colotelo, 1971a; Colotelo, 1973; Cooke, 1969; Cooke, 1971; Jones, 1970), but no attempts to elucidate the biochemical make-up of exudates associated with filamentous hyphae have been reported. The work carried out during this research shows, for the first time, details of many of the characteristics associated with the droplets found on the filamentous mycelium of Fusarium culmorum. Also presented, is evidence for the interrelationship of these droplets to the lysosomal system described by Wilson (1973). This hypothesis speculates on the possible cellular origin of the drops.

In 1973, Colotelo suggested that sclerotial exudates of Sclerotinia sclerotiorum may play a role in the process of pathogenesis and, although the ability of culture filtrates and fungal exudates to destroy host tissue has been known for some time (Brown, 1915; DeBary, 1887), tissue breakdown has never been related directly to the droplets on filamentous mycelia before now. Many of the chemical constituents of the exudate from F. culmorum are described here and the selection of which constituents to test for was based on their known or theoretical involvement in the process of pathogenesis. A number of cell wall degrading enzymes were shown to be present but as well, a heat stable component was shown to be partially involved in the process. Some evidence is given to support the possibility that this component is an oxalate.

After biochemically illustrating the potential of this exudate for tissue disintegration, the actual destructive capability of the exudate, independent of the presence of the organism, was demonstrated for four different plant tissues. This is the first time the exudate from filamentous mycelia have been linked directly to pathogenesis.

Some details of the morphology of tissue breakdown in relation to exudation were investigated. A definite loss in weight by potato tissue upon treatment with exudate was recorded, and the disintegration indicated by this, was visually demonstrated in electronmicrographs of the treated tissue. The electronmicrographs of exudate treated potato tissue bear a close resemblance to electronmicrographs of potato tissue prepared from a tuber showing symptoms of dry rot disease. It is believed that these are the first electronmicrographs showing the details of dry rot symptoms at this level.

An analysis of the breakdown product of treated tissue revealed a substantial release of galacturonic acid.

Finally, in relation to the expression of disease symptoms, evidence is given to indicate that the biochemical make-up of host tissue (in particular, the cell wall composition) plays an important role in determining the type of disease symptom expressed. Unripened tomato fruit injected with active exudate consistently resulted in internal lesions characteristic of dry rot disease, while ripened tomato fruit injected with the same exudate solution exhibited soft rot symptoms.

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APPENDICES

A and B

APPENDIX A
PROTEIN DETERMINATION

I MYCELIAL EXTRACTS	Time					
	(19-20) Hrs.	(40-44) Hrs.	(63-68) Hrs.	(110-116) Hrs.	(158-167) Hrs.	
Protein	1.7 2.2 1.9	1.9 (1.7) (2.0)	2.3 1.5 1.5	1.7 2.6 0.85		.93 1.4 1.02
$\bar{x}$ =	1.9	1.9	1.8	1.7		0.9
Std. Dev.	+ 0.21	+ 0.1	+ 0.38	+ 0.52		+ 0.40
II EXUDATE - SERIES I						
	20 Hrs.	40 Hrs.	60 Hrs.	90 Hrs.	110 Hrs.	160 Hrs.
Protein	4.4 5.6 6.6	8.2 8.8 9.1	9.4 8.0 8.9	9.8 10 6.0	6.2 8.4 5.1	5.0 5.2 3.5
	4.6	7.4	8.4		5.5	5.1
						3.3
$\bar{x}$ =	5.3	8.6	8.7	8.6	6.3	4.6
Std. Dev.	0.88	+ 0.70	+ 0.52	+ 1.8	+ 1.3	+ 0.67
						+ 0

APPENDIX A (con't.)

EXUDATE - SERIES II	Time				
	20 Hrs.	40 Hrs.	65 Hrs.	95 Hrs.	115 Hrs.
Protein	5 4.5 4.8 6.4 5.2 6.6 6.6 5.6 0.84	14 12.9 13.5 + 0.56	11.2 17.6 10.4 20.4 7 14.9 + 4.2	13.7 17.2 16.0 24 19.4 23.4 25.6 19.7 + 4.6	11 8.6 12.4 20 18.6 16.9 14.1 + 4.4
$\bar{x} =$					
Std. Dev.					

APPENDIX B

TOTAL REDUCING SUGARS DET^N.

	24 Hrs.	40 Hrs.	60 Hrs.	90 Hrs.	110 Hrs.	140 Hrs.
	19	12	8	4.6		
		10.3	7.6		1.8	
		14	14	6.8		
		18		6.3		3
		17.5				
		16.3	9.2			
$\bar{x} =$	19	14.7	9.7	5.9	1.8	3
Std. Dev.	0	± 2.9	± 2.6	± 1	0	0

CURRICULUM VITAE

GENERAL:

W. J. McPhee

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Burlington, Ontario L7L 4Y5

Born: November 22, 1937, at Toronto, Ontario

Married with three children

EDUCATION:

1961

Graduation diploma from the Ryerson Institute of Technology, in Industrial Chemistry. Toronto.

1970

B.Sc. from York University, in general Biology. Toronto.

1973

M.Sc. from York University, in general Biology. Toronto.

PUBLICATIONS:

"Effects of pesticides on the decomposition of organic matter. I: The fungistatic effect of pp'DDT on various fungi." Canada Committee on Pesticide Use in Agriculture Publication-A41-14/1970. Queens Printer for Canada, Ottawa.

"Fungal Exudates. I: Characteristics of hyphal exudates in Fusarium culmorum. Can. J. Bot. 55 (3): 358-365. (1977).

SEMINARS PRESENTED:

1. The effect of pesticides on the decomposition of organic matter. I: The effect of pp'DDT on various fungi. The 25th Ontario Universities Biological Conference. The University of Windsor, Windsor, Ontario. (1971).
2. The application of densitometry to analysis of morphological changes induced in fungal colonies. The 26th Ontario Universities Biological Conference. The University of Toronto. (1972).
3. The effect of DDT on the growth and uptake of substances by soil fungi. The Canadian Society of Plant Physiologists' meeting, York University, Toronto. (1973).
4. Mycelial exudates from Fusarium spp. and their possible role in pathogenesis. Presented at the Alberta Regional Group of the Canadian Phytopathological Society meetings, Brooks, Alberta. (1974).
5. Fungal exudates and their possible role in pathogenesis. Canadian Phytopathological Society meetings, Saskatoon, Saskatchewan. (1975).

EXPERIENCE:

1. Laboratory Technologist, Connaught Medical Res. Labs.
1961-1968. This job involved full responsibility for all chemical testing of drugs and biologicals for the purpose of quality control. I was responsible for co-ordinating the work of two technicians as well as being responsible for development of new test procedures.
2. M.Sc. student at York University. 1970-1973. During this period I worked on the effects of DDT on soil fungi. This was basically a laboratory project dealing with the growth responses of soil fungi to DDT treatment and the mechanism responsible.
3. Ph.D. student at the University of Alberta. 1973-1977.
The project has involved looking at the relationship between fungal exudates and the process of pathogenesis. Some emphasis has been on fungal physiology in an attempt to explain the origin of the exudates while a large part of the research has involved a biochemical evaluation of the exudates and the breakdown of host tissues.

TEACHING EXPERIENCE:

- 1970-71 Laboratory demonstrator in Natural Science at York University, Toronto. The course was "Principles of Life", and the supervisor Mrs. B. Mercer.
- 1971 Tutorial leader for an adult education course at Atkinson College, York University, Toronto. The course was "The Living World", and the supervisor Dr. H. Fisher.
- 1971-72 Laboratory demonstrator in Biology at York University. The course was "Biological Science 201.8", and the supervisor Dr. M.G. Boyer.
- 1973 Lecturer at Sheridan College at Brampton, Ontario. The course was "Quality Control in the Food Industry", and the supervisor was Capt. A. J. Hall.
- 1973-74
1974-75 Laboratory demonstrator in Plant Science at the University of Alberta, Edmonton. The course was "Introductory Plant Pathology", and the supervisor Dr. N. Colotelo.
- 1974-75
1975-76 Laboratory demonstrator in Biology at the University of Alberta. The course was "Evolutionary Biology", and the supervisor Ms. M.J. Turtle.

EXPERIENCE WITH THE FOLLOWING TECHNIQUES:

- (i) gas chromatography
- (ii) thin-layer chromatography
- (iii) electrophoresis
- (iv) light spectroscopy
- (v) flame photometry

- (vi) radioactive tracer technique
- (vii) densitometry
- (viii) photography (black and white processing and printing)
- (ix) scanning electron microscopy
- (x) polarography
- (xi) computer operation (particularly text processing)
- (xii) television video taping (for the purpose of preparing teaching tapes for undergraduate labs). This involved script writing, filming, and some on camera work.

COURSE WORK:

Relevant 3rd and 4th year courses:

Principles of Biochemistry
Advanced Biochemistry
Introductory Ecology
Advanced Ecology
Plant Physiology
Microbiology
Physical Biochemistry
Physical Chemistry
Radiation Biology
Molecular Biology
Animal Physiology
Solid State Physics
Under Graduate Thesis: "Some aspects of pectolytic enzyme production in cultures of Ceratocystis ulmi (Buism.) C. Moreau.

GRADUATE COURSES TAKEN:

Research seminars (seminars given each year as a graduate student)
Readings in Population Biology
Physiology and Biochemistry of Plant Pathogens

GRADUATE COURSES TAKEN: (Cont'd)

Protein Biosynthesis and Function
Microbial Physiology
Principles of Plant Pathology
Plant Pathogenesis
Advanced Plant Biochemistry
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